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SYSTEM IN CBA MICE .....
.....

UNIVERSITY.... OF ALBERTA .....

DEGREE FOR WHICH THESIS WAS PRESENTED.... Ph. D. .....

YEAR THIS DEGREE GRANTED.... 1975 .....

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ONTOGENY OF THE IMMUNE SYSTEM IN CBA MICE

by

(C) LILY YUNG LI LI

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

IMMUNOLOGY

THE PH. D. (MEDICAL SCIENCE) COMMITTEE

EDMONTON, ALBERTA

SPRING, 1975

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled ONTOGENY OF THE IMMUNE SYSTEM IN CBA MICE submitted by LILY YUNG LI LI in partial fulfilment of the requirements for the degree of DOCTOR OF PHILOSOPHY in IMMUNOLOGY.

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ABSTRACT

The ontogeny of the immune system in CBA mice was studied in terms of the acquistion of immunological functions.

To bypass the technical difficulties in monitoring the fetus *in utero*, an experimental model was designed in which single spleen colonies, derived from 12 day fetal liver cells, were transferred into irradiated syngeneic recipients. Thus the lymphopoietic and erythropoietic systems of each recipient would have their origin from a single stem cell.

This model was used to follow the onset and development of antigen recognition, humoral immune responsiveness and cell mediated GvH reactivity in CBA mice.

Recognition of a T-independent antigen POL, as judged by the ability of lymphoid cells to bind the antigen, appeared after a period of clonal expansion from a single stem cell lasting 18 to 20 days. This suggests that the complete antigen-recognition spectrum is expressed within 20 days of lymphoid development. However, recognition of a T-dependent antigen, SRC did not develop until days 30-33 of clonal expansion. Humoral immune responsiveness to these antigens as measured by antibody production, appeared after recognition, and followed the same sequence: first to POL and then to SRC. The most likely explanation for this discrepancy is that T-cells mature at a slower pace than B-

cells. Consistent with this view, T-cell mediated GvH reactivity and SRC responsiveness showed similar kinetics of development.

Immune development of fetal liver stem cells in irradiated syngeneic hosts bearing cardiac allografts did not result in tolerance towards the grafts whereas development in irradiated F1 hybrid mice induced tolerance against the alloantigens of the opposite parent. This discrepancy may have resulted from the heart allograft being a limited and localized source of antigen, and therefore not easily accessible to the developing lymphoid cells.

The development of responsiveness to antigens was faster than that to mitogens in irradiated and bone marrow reconstituted mice. The development of SRC and PHA responsiveness was shown to be entirely thymus dependent whereas that of PWM and POL responsiveness was partially influenced by the thymus. Neonatal thymectomy of bone marrow donors impaired the development of B-cells as reflected in the partial restoration of the humoral responsiveness to POL, a T-independent antigen.

The development of cells responsive to PHA and PWM was accompanied by changes in their sedimentation velocity which is directly related to cell size. In the early stages of development, cells were larger, and became progressively smaller as they matured. Cells responsive to PHA and PWM could not be distinguished on the basis of size differences because there was considerable overlap in the sedimentation

velocity distribution profiles, and T-cells respond to both mitogens.

PHA responsiveness was detectable in fractionated spleen cells within 50 days of repopulation. To account for this observation, a hypothesis was proposed that this is due to the separation of potential reactive cells from suppressor cells. The presence of the latter may account for the high susceptibility to tolerance induction of the young and the establishment of self-tolerance.

PREFACE

This dissertation describes an experimental model which allows the study of the ontogeny of immunocompetent cells *per se*, and the spplication of it to follow the development of some aspects of immunological functions in CBA mice.

Some of the results in Chapter IV have been published in the European Journal of Immunology under the title "Ontogeny of the murine immune system: development of antigen recognition and immune responsiveness".

Part of the work in Chapter VI, namely 'Tolerance induction in the heart allograft model' and 'Adoptive transfer of tolerance to Balb/c heart allografts' was done in collaboration with Dr D.W.Jirsch and Dr N.Kraft respectively.

Studies on the development of mitogen responsiveness described in Chapter VII were performed in collaboration with Dr G.Tridente.

ACKNOWLEDGEMENTS

I am indebted to many persons in the department of Immunology and elsewhere for their interest and assistance in my work. I would like to thank particularly:

Dr. Erwin Diener, for his advice and encouragement throughout the course of my work.

Drs. Rodney E. Langman and Kwok-choy Lee, for their invaluable discussions and criticisms in the preparation of the manuscript.

Mr. David W. Lloyd, for his advice and help in the compilation of the manuscript by computer.

This work was supported by the Medical Research Council of Canada. The author gratefully acknowledges the receipt of a Medical Research Council Studentship from 1973-1974.

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LIST OF ABBREVIATIONS

The nomenclature for immunoglobulins and chemical compounds conforms to that recommended by the World Health Organization and IUPAC respectively. Other abbreviations and symbols used included:

ABC	antigen binding cells
A-cells	adherent cells
AFC	antibody forming cells
B-cells	bursa-derived or non thymus-derived cells
BSA	bovine serum albumin
CBF1	(CBA x Balb/c) F1 hybrid
CFU	spleen colony forming unit
DNA	deoxyribonucleic acid
EBSS	Eisen's balanced salt solution
FCS	fetal calf serum
GvH	graft versus host
H-2	histocompatibility locus in mice
Ig	immunoglobulins
MBLA	mouse-specific bone marrow-derived lymphocyte antigen
MEM	minimal essential medium

MON-	monomeric Salmonella flagellin
N	non-thymectomized
nTx	neonatally thymectomized
PBS	phosphate buffered saline
PFC	plaque forming cells
PHA	phytohemagglutinin
POL	polymerized Salmonella flagellin
PWM	pokeweed mitogen
RES	reticuloendothelial system
RFC	rosette forming cells
S.E.	standard error of the mean
SRC	sheep red cells
TCA	trichloroacetic acid
T-cells	thymus-derived cells
TL	thymus leukemia
TNP	trinitrophenol
Tx	thymectomized at 15 days of age
θ	theta

CHAPTER I

INTRODUCTION

The central theme of this chapter is the functional aspect of development in the immune system. The general characteristics of adaptive immunity in terms of humoral antibody production and cell-mediated transplantation immunity, in fetal and neonatal animals are briefly reviewed. Since lymphoid organs and cells constitute the immune apparatus, the origin of lymphocyte precursors, their development, and the role of primary organs during ontogeny are topics which are considered in some detail. The influence of the reticuloendothelial system (RES) on the development of immunological functions is also discussed because of the close anatomical and functional relationship between the immune system and the RES.

A. Adaptive Immunity in Fetal and Neonatal Animals

1. Active Immunity in Fetal and Neonatal Animals

It is technically difficult to monitor the fetus *in utero*, especially in small mammals like the laboratory rodents. As a result, investigations on the development of immunological functions in smaller mammals have been restricted to the post partum period.

In bigger mammals, major experimentation on the fetus *in utero* has been pioneered by Schnickel and Ferguson (1953) who found that surgical penetration of the gravid uterus of

the sheep was possible quite early in gestation without jeopardising the chances of normal delivery. They showed that as early as 80 days post conception, fetal lambs (with a gestation period of 150 days) were already able to reject skin allografts. Their results were confirmed by later studies of Silverstein, Prendergast and Kraner (1964). These workers also looked at the ability of fetal lambs to produce antibodies against several antigens. From their results, they concluded that humoral immunity following antigenic stimulation develops in a stepwise fashion. Thus as early as 38 to 40 days of gestation, fetal lambs could produce antibodies against bacteriophage øx174 but not against ferritin or ovalbumin until 66 and 120 days of gestation respectively (Silverstein et al. 1963; Silverstein, Parshall & Uhr, 1966). The stepwise development of humoral immunity against different antigens during gestation also appears to be true in fetal Rhesus monkeys (Silverstein & Kraner, 1965). They interpreted this phenomenon as probably due to the sequential appearance of immunocompetent cells with different recognition specificities. Immune ontogenic studies in utero have also been performed in other species, including rabbits (Sterzl & Trnka, 1957; Porter, 1960), pigs (Binns, 1967) and cattle (Pennestad & Borg-Petersen, 1962).

Compared to other eutheriotic mammals, marsupials are born at a relatively immature stage and continue a fetal-like development in a maternal abdominal pouch. According

to Block (1964), the blood and blood forming tissue of an opossum at birth correspond in development to a 10-day mouse embryo. Thus the immunological competence of marsupials can be observed starting from an early stage of ontogeny. Rowlands, La Via and Block (1964) showed that as early as 5 days after birth, opossums can respond to bacteriophage stimulation with antibody production. LaPlante et al. (1969) found that skin allografts survived for at least 80 days when grafted on pouch-young less than 12 days after birth, but were rapidly rejected by older opossums.

Birds, too, offer a model for studying the early ontogenic events, with the additional advantage that uniform inbred strains of chickens are available. Murphy (1913) first demonstrated that foreign grafts of mammalian and avian tissues, as well as a variety of tumors are not rejected by the chick embryo before the 10th day of incubation. Using the splenomegaly assay of Simonsen (1957), Solomon (1961) showed that spleen cells from 5 day old chickens are competent to induce splenomegaly in 8 day old embryos. Using resistance to the induction of splenomegaly as an index of immunocompetence, Isacson (1959) showed that splenomegaly was greatest in embryos injected with allogeneic cells at 13 days of incubation. Thereafter, a decrease in splenomegaly following cell injection was observed in older embryos. One day old chicks were almost completely resistant to the induction of splenomegaly (Solomon & Tucker, 1963). Two day old chicks were able to

reject skin allografts (Solomon, 1963). More rapid rejection was observed when the chickens were injected intravenously with allogeneic blood at 19 days of incubation (2 days before hatching) and subsequently challenged with skin grafts from animals of the same genotype as the blood donors. Therefore it seems that chickens can be primed against alloantigens before hatching although the ability to participate in cell-mediated immunity does not appear until shortly after hatching. As for humoral immunity, Solomon (1966) has shown that 17 and 19 day old chick embryos injected with 5 to 10 x 10^9 goat red cells are able to produce opsonising antibodies at 3 and 5 days after hatching. The number of antibody forming cells to sheep red cells (SRC) reached a maximum in chickens immunized 5 days after hatching (Solomon, 1968). Thus as in cell-mediated immunity, the ability of chickens to produce specific antibodies following antigenic stimulation is also developed just after hatching. Comparable results have been obtained in ducks and turkeys (Hraba & Hasek, 1956).

In the mouse, transplanation immunity as measured by rejection of skin allografts was demonstrable in newborns (Steinmuller, 1961; Boraker & Hildeman, 1965), although resistance to tumor growth was low (Attia, Deome & Weiss, 1965; Boraker & Hildeman, 1965). Simonsen (1957) demonstrated that 4 day old mice were resistant to the induction of splenomegaly by allogeneic spleen cells. Dalmasso *et al.* (1963) and Argyris (1965) found a strain

variation in the time of onset of the ability to induce graft versus host (GvH) responses. Similar strain variations were observed with respect to the onset of antibody production to antigenic stimulation (Hechtel, Dishon & Braun, 1966; Playfair, 1968). Data from various laboratories point unanimously to the fact that at birth, mice are incapable of producing a humoral response in vivo against SRC antigens (Wigzell & Stjernsward, 1966; Playfair, 1968; Arrenbrecht, 1973). A progressive increase in responsiveness is observed between 1 to 2 weeks after birth.

The literature on the ontogeny of adaptive immunity suggests that the development of immunocompetence in birds as well as in mammals is indicated by a progressive increase in the variety and magnitude of immune responses that the animal can exhibit. The data also suggest that both cell mediated and humoral immunity develop at about the same time (Solomon, 1971). In the majority of animals studied, the ability to participate in adaptive immune responses develops shortly after birth, although in fetal sheep and monkeys this occurs during the first half of gestation (Silverstein, Parshall & Prendergast, 1967).

2. Effects of Maternal Antibodies on the Offspring

It is well documented that maternal antibodies are transferred into fetal and neonatal animals. This passage may occur via the yolk sac, the placenta or through colostrum absorption, depending on the species. A summary

TABLE 1 Routes of maternal antibody transfer in different species

Species	Routes of transfer
ungulates	colostrum
dog	colostrum & placenta
rat, mouse	colostrum & yolk sac
rabbit, guinea pig, bird	yolk sac
primates	placenta

of the routes of transfer of maternal antibodies in birds and mammals is given in Table 1.

There is selectivity in the class of immunoglobulins transmitted via different routes in different species. In primates, which receive maternal antibodies entirely through the placenta, only IgG (7S) is readily transferred (Cann et al. 1951; Gitlin et al. 1964). In birds there is selective discrimination against IgM amongst the antibodies transmitted via the yolk sac (Malkinson, 1965). However, in rabbits, Hemmings and Jones (1962) showed that IgM hemagglutinin is transmitted in addition to IgG. In rats and mice, maternal antibodies, which are transmitted into the young through colostral-intestinal absorption, contain no IgM or IgA (Morris, 1969; Fahey & Barth, 1965). The colostrum constitutes the sole source of maternal antibodies in ungulates and IgG, IgM and IgA are transmitted non-discriminantly (Halliday, 1965; Klaus, Bennett & Jones, 1969; Porter, 1969).

Since antibody production does not begin until some time after antigenic confrontation even in adult animals, maternal antibodies provide passive immunologic protection for the neonate during its immediate extrauterine life. However, it has been demonstrated that maternal antibodies can also suppress active antibody production by the neonate (Osborn, Dancis & Julia, 1952; Uhr & Baumann, 1961), thereby causing unresponsiveness at that stage of development. Piglets provide a useful model for the study of active

antibody production in the absence of maternal antibodies because they receive maternal antibodies entirely through the colostrum and can be reared in a germ-free environment on diets free of colostral antibodies. Sterzl et al. (1965) found that in conventionally reared piglets, the PFC response to SRC did not appear until 14 days after birth, whereas colostrum-deprived piglets injected with SRC 1 day after birth were found to have PFC 3 days later. However, studies of Hoerlein (1957) and Segre and Kaeberle (1962) showed that non-immune colostrum can enhance antibody production in colostrum-deprived piglets. This enhancing effect seems to be due to the presence of IgM (Locke, Segre & Myers, 1964). The results of Hoerlein are difficult to interpret since 4 antigens were administered at the same time and the effect of antigenic competition would reduce the antibody responses as, in fact, was shown by Kim, Bradley and Watson (1967).

The nature of inhibition by maternal antibodies is still not clear. Two mechanisms have been proposed. Passively administered antibodies may opsonize antigens thus reducing the immunogenic stimulus (Dixon, Jacot-Guillarmod & McConahey, 1967; Walker & Siskind, 1968). Alternatively, passively administered antibodies may inhibit antibody production by a feedback-like action at the level of immunocompetent cells (Moller, 1968; Graf & Uhr, 1969).

3. Tolerance Induction

Under certain conditions, antigen administration does not lead to immune induction but to specific unresponsiveness or tolerance. Thus the failure of young animals to respond to an antigenic stimulation may be due to the induction of tolerance. Billingham, Brent and Medawar (1953) showed in their classical experiment that immunologic tolerance to homografts in inbred mice can be produced by injections of cells from the prospective donor strain into the prospective graft recipients during late fetal life. Although tolerance induction is not restricted to embryonic life (Dixon & Maurer, 1955; Martinez et al. 1959; Simonsen, 1960), data from different laboratories have provided evidence that it is much easier to induce tolerance in young animals than in adult animals (Howard & Michie, 1962; Brent & Gowland, 1961, 1963).

Three explanations have been suggested for the ease of tolerance induction in the young. Firstly, during immune maturation, the animal may pass through a 'tolerance responsive period' during which encounter with antigens would give rise to specific unresponsiveness (Billingham et al. 1959). Secondly, the dose of antigen required for tolerance induction in the young may be lower than that in the adult (Smith & Bridges, 1958; Brent & Gowland, 1962; Nossal, 1974). Finally, the low phagocytic activity of the young may render antigens less immunogenic (Nossal & Mitchell, 1966; Mitchell & Nossal, 1966). In support of

this last point is the finding that in adults, susceptibility to tolerance induction is correlated with the phagocytic activity of the animal (Das & Leskowitz, 1974).

It is not known whether tolerance to self and non-self antigens is induced and maintained by the same mechanism. On the basis that blood cell chimerism occurs in synchorial cattle twins (Owen, 1945), Burnet and Fenner (1949) proposed that self tolerance resulted from elimination or inactivation of self-reactive lymphoid cells upon encountering autoantigens during ontogeny. Likewise, extrinsic antigens brought into contact with immature lymphoid cells would be regarded as 'self' and thus exhibit tolerogenic rather than immunogenic properties. The availability of tetraparental mice (Mintz, 1962) has provided an excellent model for the analysis of tolerance in the fetus. In a tetraparental mouse, there exists a state of mutual tolerance between two populations of lymphocytes which have different histocompatibility antigens. Wegmann, Hellstrom and Hellstrom (1971) have shown that the state of tolerance in tetraparental mice differs from that in F1 hybrids. They found that washed lymphocytes from tetraparental mice were able to generate a mixed lymphocyte reaction <u>in vitro</u>. Lymphocytes from F1 animals did not demonstrate such reactivity under similar conditions. These results were used to argue that tolerance in chimeras represents an acquired state of suppressed reactivity. Using another experimental model, Triplett (1962) has

previously shown that tolerance to self antigens is an acquired state. In his experiment, the adenohypophysis was removed from frogs at the early larval stage, propagated in allogeneic larvae and reimplanted into the host of origin at a later stage of maturation. The organ was rejected despite the fact that it was 'self'. However, there is the possibility that there may have been a carry over of tissue from the allogeneic intermediate host thus eliciting a homograft rejection. Triplett concluded that self-tolerance is an acquired state that suppresses the normal expression of anti-self activity as it is generated. Consistent with this view is the presence in normal subjects of cells capable of binding self antigens (Ada & Cooper, 1971; Yung et al. 1973; Bankhurst, Torrigiani & Allison, 1973), as well as of autoantibodies (Whittingham et al. 1969). According to the current consensus, suppression of self reactive lymphocytes is mediated by serum blocking factors (Hellstrom and Hellstrom, 1969) which have been suggested to be antigen-antibody complexes (Diener, Feldmann & Armstrong, 1971; Sjogren et al. 1971; Baldwin, Price & Robins, 1972; Wright et al. 1973).

B. Development of Lymphoid Organs and Cells

1. Lymphoid Organs

In vertebrates, the main lymphoid organs include the thymus, the bursa of Fabricius (in birds only), lymph nodes, spleen and organized lymphoid tissue of the alimentary

canal: tonsils, adenoids, Peyer's patches, appendix and in the rabbit, sacculus rotunda. On the basis of their functional attributes, lymphoid organs can be distinquished into primary and secondary.

Primary organs are those which exhibit autonomous lymphopoiesis and intense antigen independent cellular proliferation. In birds, two primary lymphoid organs have been described: the thymus and the bursa of Fabricius. In mammals, the thymus has all the characteristics of a primary organ. However, attempts to locate a bursal-equivalent in mammals so far have yielded equivocal results.

Studies in animals including chicken, mouse, hamster, rat, dog and man show that the first lymphoid development occurs in the thymus (Solomon, 1971). In the mouse, lymphopoietic activities are found in the thymus by day 14 of gestation while the spleen and nodes do not develop lymphoid structures until after birth (Ball & Auerbach, 1960; Raff & Owen, 1971a). In chickens, 3 days before hatching, only the thymus and the bursa of Fabricius have identifiable lymphoid tissue (Papermaster & Good, 1962).

In secondary lymphoid organs, lymphopoiesis depends on the presence of primary organs (Auerbach, 1963a) and cellular proliferative activities are antigen dependent resulting in the production of specific antibodies and of sensitized lymphocytes.

2. Origin of Lymphoid Precursor Cells

Evidence for the origin of lymphoid cells comes from cell-marker studies in chimeric animals. It has been shown that adult mouse bone marrow when injected into lethally irradiated syngeneic recipients can reconstitute the hemopoietic and lymphoid compartments of the latter (Ford et al. 1966). These cells are referred to as hemopoietic stem cells and they have been defined as a class of pluripotential stem cells having self renewal properties (Barnes & Loutit, 1967). Studies by Wu et al. (1968) further showed that single stem cells were able to differentiate along lymphoid, erythroid, granulocytic and megakaryocytic lines.

In mice, the bone marrow is not the only site where stem cells are found. The adult spleen and fetal liver represent rich sources of stem cells. However, the ontogenic origin of stem cells is the yolk sac. Evidence for this comes from the work of Moore and Owen (1967a). These workers injected yolk sac cells from male chickens into irradiated female chicks at 14 days of incubation. Two days following hatching, chromosome analysis of host tissues showed the presence of donor cells in the thymus, spleen, bone marrow and bursa. Since cellular exchange occurred between partners of parabiosed chickens (Moore & Owen, 1965, 1966, 1967b), these workers concluded that there is migration of stem cells from the yolk sac via blood into the hemopoietic tissues.

It has also been shown that mouse yolk sac cells can repopulate the lymphoid and myeloid tissues of lethally irradiated adult hosts (Tyan, 1968; Tyan & Herzenberg, 1968; Moore & Metcalf, 1970). Moreover, cells from yolk sac or from fetal liver were found to contain precursors of cells involved in cell-mediated immunity (Tyan & Cole, 1963, 1964, 1966; Tyan, Cole & Nowell, 1966), as well as in immunoglobulin production (Tyan, Cole & Herzenberg, 1967; Tyan & Herzenberg, 1968).

It has been shown that stem cells in the yolk sac and liver of fetal mice less than 12 days of gestation differ from those found in late fetal liver or adult bone marrow. They can be differentiated according to size (Haskill & Moore, 1970), density (Moore, McNeill & Haskill, 1970), proliferative activity (Micklem & Loutit, 1966; Kubanek et al. 1969), sensitivity to gamma irradiation (Siminovitch, Till & McCulloch, 1965), suicidal levels of tritiated thymidine (Becker et al. 1965) and seeding efficiency to the spleen (Silini, Pons & Pozzi, 1968; Moore & Metcalf, 1970). Moreover, Haskill and Moore (1970) showed that stem cells of embryonic type are absent from adult resting marrow. Haskill (1971) has further shown that early fetal liver stem cells give rise to cells with characteristics resembling adult bone marrow stem cells.

The maturation of lymphoid cells is much influenced by primary lymphoid organs, namely the thymus and the bursa of Fabricius.

3. Role of Primary Lymphoid Organs on Lymphocyte Maturation

a. Thymus

Studies in various species have shown that the first lymphoid development occurs in the thymus. Auerbach (1964) has demonstrated that lymphoid differentiation can occur when prelymphoid thymus rudiments from 12 day old mouse embryos are grown in tissue culture. Originally this result was thought to indicate that lymphoid stem cells originate from the thymus. Later work by Moore and Owen (1967c) provided evidence that stem cells of the thymus originate from the large basophilic cells found in the yolk sac. It is likely that stem cells of the adult thymus are of bone marrow origin. Thus Micklem et al. (1966) found bone marrow cells with a distinct chromosome marker proliferating in the thymus of irradiated mice.

The fate of thymic lymphocytes has been followed by in situ labeling of young rat thymus with tritiated thymidine (Weissman, 1967). Appreciable numbers of highly labeled cells were found in the diffuse cortex of lymph nodes and in the peripheral white pulp of the spleen, but no labeled cells were found in the bone marrow or intestinal mucosa. The results suggested that migrant cells from the thymus comprise a major portion of the total lymphocyte population in lymph nodes and spleen. The contribution of thymus lymphocytes to the developing lymph nodes and Peyer's patches has been demonstrated by the studies of Raff and Owen (1971b), Owen, Hunter and Raff (1971) and Joel, Hess

and Cottier (1971).

Under normal conditions, or in irradiated recipients, the development of full immunocompetence from lymphoid progenitors is dependent upon the presence of the thymus (Miller, 1961; Martinez et al. 1962; Metcalf, 1964). The role of the thymus in the development of immunocompetence has been deduced by examining the effects of thymectomy on immunological reactivities. Miller (1961, 1962, 1964) and Martinez et al. (1962) have shown that neonatal thymectomy causes severe impairment of immunocompetence and the thymectomized animals suffer from runting disease, lymphocytopenia and cellular depletion in their secondary lymphoid organs. In rats, Gowans and Knight (1964) have shown that neonatal thymectomy reduces the number of recirculating lymphocytes. Cellular deficiency caused by neonatal thymectomy has been found to occur in the thymus-dependent areas of peripheral lymphoid organs, namely the periarteriolar sheaths in the spleen and the paracortical areas in lymph nodes (Parrott, de Sousa & East, 1966). On the other hand, germinal centre formation and plasma cell production are not affected by thymectomy (Parrott, de Sousa & East, 1966). Similar results were obtained in chickens (Warner & Szenberg, 1962; Jankovic & Isakovic, 1964). The severity of the effect of neonatal thymectomy varies amongst different species. This is thought to be caused by their different kinetics of lymphoid development and peripheralization of lymphocytes (Archer, Papermaster &

Good, 1964).

The damaging effects of thymectomy become less pronounced as the age at which the operation is performed increases (Dalmasso et al. 1963). Thus mice thymectomized in adulthood do not manifest deficiency in immune responsiveness until 6 to 12 weeks after the operation or even later (Taylor, 1965; Metcalf & Brumby, 1966). The effect of adult thymectomy is best demonstrated in thymectomized irradiated mice, which show a marked impairment of lymphoid recovery (Auerbach, 1963b; Miller, Doak & Cross, 1963). These results indicate that the thymic influence on lymphopoiesis extends into adult life.

Removal of the thymus affects the development of responsiveness in cell-mediated immunity (Miller, 1961; Martinez et al. 1962), as well as humoral antibody production to most antigens (Miller & Osoba, 1967; Claman & Chaperon, 1969).

Restoration of immunocompetence in thymectomized mice may be achieved by syngeneic (Miller, 1962a; East & Parrott, 1964) or allogeneic (Dalmasso et al. 1963) thymus grafts. It is interesting to note that these grafts were repopulated with host rather than donor cells (Dukor et al. 1965). This suggests that restoration is not entirely dependent upon lymphoid cells from thymic grafts but that the thymic epithelium can provide the necessary environment for lymphocyte development.

Thymus grafts enclosed in cell-impermeable diffusion

chambers have been found to bring about restoration of immune responsiveness in thymectomized mice (Osoba & Miller, 1963), and suggests that the thymus may secrete a substance which promotes immunological maturation. Results from various laboratories support the idea that the thymus may exert a humoral influence on the maturation of lymphoid cells in peripheral organs (Law & Agnew, 1968; Stuttman, Yunis & Good, 1969; Small & Trainin, 1971; Lonai et al. 1973).

b. Bursa of Fabricius

In chickens, the bursa of Fabricius is another primary lymphoid organ (Ackerman & Knouff, 1959, 1964; Ackerman, 1962). This organ becomes lymphoid at a later stage of development than the thymus. Chromosome marker studies in parabiosed chicken embryos have shown that lymphocytes within the bursal follicles are derived from migrant, blood-borne stem cells of yolk sac origin (Moore & Owen, 1966) and may be identical to those seeded into the thymus (Moore & Owen, 1967c).

Glick, Chang and Jaap (1956) first demonstrated an association between the bursa of Fabricius and the development of immune responses when they found that chickens surgically bursectomized 12 days after hatching had a greatly reduced capacity to produce agglutinins against Salmonella typhimurium. Hormonal bursectomy by injection of testosterone into chickens on day 5 of incubation has been

shown to depress the antibody forming capacity of the treated birds (Mueller, Wolfe & Meyer, 1960; Warner, Szenberg & Burnet, 1962). In contrast to thymectomy, bursectomy has been found to have no effect on the capacity of chicks to reject skin homografts (Warner et al. 1962; Aspinall et al. 1963). From the results of their studies, Warner and Szenberg (1962) defined two types of lymphocytes: one bursa derived (termed B-cells) and the other thymus derived (termed T-cells). Studies on the effect of bursectomy, especially during late embryogenesis, are consistent with the view that the bursa is the site of maturation of the immunoglobulin producing cell line (Cooper et al. 1969). Little work has been done on restoration of bursectomized birds with bursal grafts although there are reports that the antibody forming capacity of bursectomized chicks is restored by bursal homografts (Isakovic et al. 1963) or bursal grafts enclosed in cell-impermeable diffusion chambers (Jankovic and Leskowitz, 1965). However, Dent et al. (1968) showed that antibody formation in these restored chicks might be an artifact due to bacterial contamination of bursas of newly hatched chicks which were used for grafting since bursas of 19 day embryos, which presumably were not contaminated, failed to exert any restorative effect.

The discovery in birds of a second primary lymphoid organ, the bursa of Fabricius, has led to speculation about a similar organ in mammals. The study of immunological

deficiency diseases in humans provides convincing evidence for immunological dissociation between thymus-dependent T-cells and those dependent on other central lymphoid tissues (Janeway et al. 1967; Cooper, Gabrielsen & Good, 1967). Perey, Cooper and Good (1968) have shown that the intestinal lympho-epithelial tissue in rabbits is necessary for the maturation of precursors of antibody-forming cells and they suggest that it may be a candidate for a mammalian bursal equivalent. Recently, Chapman, Johnson & Cooper (1974) also found histological evidence that lymphocytes first appear in the Peyer's patches of fetal pigs. However, the intestinal lymphoid tissue reacts to antigenic stimulation with germinal centre formation - a characteristic of secondary organs.

Regardless of the existence of a discrete bursal-equivalent, mammals also exhibit a dichotomy within the lymphocyte population which is reminiscent of that described in chickens. Davies et al. (1967) found that thymus derived cells do not produce antibodies. The elegant experiment of Mitchell and Miller (1968) has provided conclusive evidence that bone marrow derived cells are precursors of antibody forming cells. Using chromosome marked cells in the same experimental model, Nossal et al. (1968b) further substantiated this view. Functionally, therefore, bone marrow derived cells in mice resemble bursa derived B-cells in chickens. For convenience, the two classes of lymphocytes in mammals have been referred to as thymus

derived T-cells and bone marrow derived B-cells (Roitt et al. 1969).

4. Development of Immunocompetent Cells

The two classes of lymphocytes, namely T- and B-cells, have different immunological functions. T-cells have been shown to be involved mainly in cell-mediated immune responses such as delayed hypersensitivity and homograft rejection. B-cells, on the other hand, are responsible for antibody production in humoral responses. In most instances, humoral responsiveness to antigens requires collaboration between T- and B-cells (Miller & Osoba, 1967; Claman & Chaperon, 1969).

Beside immune functional differences, B- and T-cells can be distinguished by other characteristics such as surface antigenic markers, physical attributes and responsiveness to phytomitogen activation in vitro. In the following sections, major studies in the development of B- and T-cells in mice using surface markers and functional characteristics are reviewed.

a. Lymphocyte surface markers

Antigenic markers on the surface of murine lymphocytes can be divided into two categories. Firstly, there are markers which are present on both B- and T-cells such as the major histocompatibility antigens (H-2) and immunoglobulin molecules. Secondly there are other markers which are found to be lymphocyte class specific. The alloantigen theta (θ)

is found on T-cells and cells from the brain and epithelial tissue but not B-cells (Reif & Allen, 1964; Raff, 1969). Another T-cell specific marker is the thymus leukemia (TL) antigen (Old, Boyse & Stockert, 1963). B-cell specific markers include the MBLA antigen (for mouse-specific bone marrow-derived lymphocyte antigen, Raff, Nase & Mitchison, 1971), receptors for antigen-antibody-complement complexes (Bianco, Patrick & Nussenzweig, 1970) and receptors for the Fc fragments of immunoglobulins (Basten, Sprent & Miller, 1972).

The distribution of surface markers on B- or T-cells is different. Aoki et al. (1969) have shown that thymocytes have fewer H-2 antigens on their surface than do B-cells. Similar results are obtained in the case of surface immunoglobulin molecules (Raff, Sternberg & Taylor, 1970; Nossal et al. 1972). Furthermore, the distribution of surface markers also varies with the different stages of maturation of the lymphocytes (Aoki et al. 1969; Owen & Raff, 1970; Schlesinger & Yron, 1970; Raff & Owen, 1971a; Takahashi et al. 1971; Ryser & Vassalli, 1974). It is thus possible to follow the development of B- and T-cells using lymphocyte surface markers.

b. Expression of surface characteristics during ontogeny

Although B-cells have a high density of surface immunoglobulin, these surface markers are either absent or present in very low density on bone marrow precursors

(Basten et al. 1971). Using ^{125}I-labeled anti-mouse immunoglobulin and autoradiography, Nossal and Pike (1972) have shown that liver cells of CBA embryos at 12.5 to 13.5 days of gestation do not become labeled. This is consistent with the interpretation that B-cell precursors have few surface immunoglobulin molecules. By 16.5 days of gestation, immunoglobulin-positive B-cells were detectable in the spleen and in circulation, and later also in the liver. Furthermore, specific antigen binding cells (ABC) were found in the spleens of 16-day fetuses. In a more detailed study, Spear et al. (1973) have also demonstrated the presence of B-cells, T-cells and antigen binding cells for 3 independent antigens (SRC, TNP and rabbit erythrocytes) in 16-day fetal spleens of Swiss L mice. These ABC were found to occur with the same frequency and a similar range of avidity as those in adult animals. These data suggest that in B-cells, antibody diversification is completed within a period of 16 days of development.

Since more than 50% of the ABC to SRC in normal spleens have been shown to carry the θ-antigen (Greaves & Moller, 1970; Bach & Dardenne, 1972), it is conceivable that the SRC binding cells demonstrated by Spear et al. (1973) in 16-day fetal spleens consisted of both T- and B-cells. Dwyer and MacKay (1970) have found ABC to Salmonella flagellin antigen in early human fetal thymuses at 20 to 22 weeks of gestation at a frequency higher than that observed in the fetal spleen or in adult tissues. In a later study, Dwyer, Warner and

MacKay (1972) have shown that ABC are present in the fetal thymus of various species. They suggested that the thymus was probably the site for the generation of antibody specificities in a non-random, genetically programmed fashion.

Schlesinger and Hurvitz (1969), and Owen and Raff (1970) studied the differentiation of thymus derived lymphocytes in mice using θ and TL markers. They could not find θ or TL on cells from yolk sac or liver of 11 day old embryos. However, by days 16 and 18 of gestation, fetal thymus cells were shown to be positive for θ and TL. A similar sequence of changes was observed in organ cultures of 14 day embryonic thymus rudiments inside cell-impermeable millipore chambers which were placed onto the chick chorioallantoic membrane (CAM). Thus the possibility that θ and TL positive cells in the thymus are migrant cells can be excluded. The results also suggest that T-cell precursors have few θ or TL surface antigens, or none at all.

Spear et al. (1973) have found θ-positive cells in 16 day fetal spleens of Swiss L mice. This is in accord with the findings that cells from the thymus seed into peripheral lymphoid organs (Weissman, 1967; Davies, 1969; Raff & Owen, 1971b). Aoki et al. (1969) compared the quantitative distribution of alloantigens on thymocytes and peripheral T-cells. Their results show that peripheral T-cells have more H-2 antigens, less θ, and no TL antigens. Leckband and

Boyse (1971) have presented evidence that a minor population of thymocytes has an alloantigen distribution similar to that of peripheral T-cells. This subpopulation is found to be resistant to the effects of corticosteroids (Levine & Claman, 1970) and is thought to consist of mature T-cells ready for emigration from the thymus. The cells in this subpopulation also behave like peripheral T-cells in (a) GvH reactivity (Blomgren & Andersson, 1970); (b) helper function in the humoral response to SRC (Cohen & Claman, 1971); and (c) PHA responsiveness (Blomgren & Svedmyr, 1971). Therefore, these results are in agreement with the idea that T-cell precursors, upon entry into the thymus, undergo proliferation and differentiation as indicated by sequential expression of θ, TL and H-2 antigens. Maturation is accompanied by a loss of TL, a decrease of θ, and a concomitant gain in H-2. Studies in rats by Order and Waksman (1969) have also described the differentiation of surface antigens on cells repopulating the irradiated thymus. The significance of these changes in the distribution of surface antigens on lymphocytes is still unknown.

c. Expression of immunological functions.

Immune responsiveness <u>in vivo</u> represents the outcome of complex interactions between different tissues and cells. Thus unresponsiveness to foreign antigens in the fetus or neonate may result from immaturity of its lymphoid cells or

its failure to provide a favourable environment for functional expression of immunocompetent cells. This thesis has been tested using the adoptive transfer of cells into irradiated hosts. Dixon and Weigle (1959) and Harris et al. (1962) were able to show that antigen stimulated cells from neonatal rabbits are able to synthesize antibodies upon transfer into irradiated adult hosts. Makinodan and Peterson (1962), however, found that spleen cells from one week old mice were deficient in the capacity to produce antibodies when transferred into irradiated adults. The ability of a neonatal environment to support antibody production has also been investigated and the results are consistent with the view that a neonatal environment can support on-going antibody synthesis but not the initiation of antibody production (Dixon & Weigle, 1957; Nossal, 1959; Mark & Dixon, 1963).

Since antibody production to most antigens depends on T-B cell collaboration (Miller & Osoba, 1967; Claman & Chaperon, 1969), unresponsiveness of the young to such antigens may result from immaturity of either T- or B-cells. Using the adoptive transfer system, Chiscon and Golub (1972) studied the ontogeny of murine B- and T-cells in a system that allowed fetal cells to co-operate with the adult counterparts in the SRC response. Their results show that B-cell activity is detectable in the fetal liver at 16 days of gestation and in the spleen shortly before birth. T-cell function in antibody production to SRC first appears in the

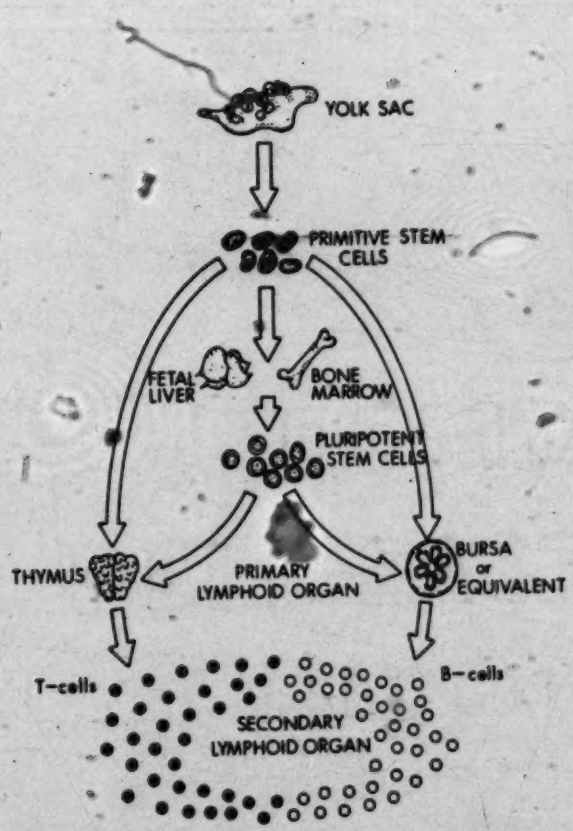

Figure 1 Ontogeny of thymus-derived (T) and non thymus-derived (B) lymphocytes.

thymus 2 days after birth and not in the spleen until 4 days after birth. Studies in tissue culture by Spear and Edelman (1974) confirmed the late appearance of T-cell function in the antibody response to SRC. Studies on the development of GvH activity in mice (Cohen et al. 1963; Sosin, Hilgard & Martinez, 1966) have demonstrated that cells able to elicit a GvH response are present in the thymus at birth but absent from the spleen and liver. These results together with those obtained using alloantigen markers are in agreement with the idea that a subpopulation of mature T-cells first appears in the thymus and later in pheripheral lymphoid organs (Owen & Raff, 1970; Raff & Owen, 1971b; Joel, Hess & Cottier, 1971). In rabbits, Knight and Ling (1969) have shown that cells in the newborn thymus could respond to staphylococcal filtrate by proliferation but no such activity could be detected in spleen cells until 22 days after birth. It has been suggested that differentiation of T-cells may also take place in pheripheral organs (Stuttman, Yunis & Good, 1969; Cantor, 1972).

The main features in the development of immunocompetent cells are summarized in Fig.1. The lymphocyte precursors are derived from some primitive stem cells of the yolk sac. These yolk sac cells undergo differentiation into hemopoietic stem cells of similar characteristics as those found later in other hemopoietic tissues. Lymphoid cells do not follow the same pathway of differentiation. Instead, they develop into T- and B-cells, the maturation of which is

T-dependent and T-independent respectively. In birds, the maturation of the B-lineage is bursa dependent. The development of B- and T-cells is marked by the expression of cell surface antigens and the subsequent changes in their distribution pattern. In mice, there is evidence that both B- and T-cells appear at the same time of development although the functional expression of B-cells seems to occur earlier.

d. The reticuloendothelial system (RES) and immune ontogenesis

Besides the lymphoid tissues, an important component of the immune system is the reticuloendothelial system which consists of phagocytic cells - the macrophages and the reticular dentritic cells. Studies in adult animals have shown that antigens administered *in vivo* may localize in the lymphoid follicles, medullary macrophages of lymph nodes and the white pulp of the spleen (Nossal *et al*. 1966). Detailed studies using high resolution autoradiography have revealed that antigen retention in lymphoid follicles is a property of reticular dentritic cells (Nossal *et al*. 1968a). Since antigen processing and presentation is an essential step in immune induction, immaturity of phagocytic cells may be one of the many factors responsible for the absence of immunological reactivity in fetal and neonatal animals. Evidence in support of this contention is provided in the studies by Argyris (1968, 1969), Nossal and Mitchell (1966)

and Bendinelli, Senesi and Falcone (1971). Studies by Reade (1968) showed that phagocytosis in fetal and neonatal rats is in part a function of the level of opsonins in the serum.

Different cells in a lymphoid organ are arranged in a special anatomical arrangement for maximal efficiency in immune induction (Nossal & Ada, 1971). Thus the lack of immune reactivity in the young may also result from the absence of proper anatomical architecture in their lymphoid organs as suggested by the work of Williams and Nossal (1966a, 1966b) in neonatal rats. They reported a close temporal relationship between the degree of maturation of the antigen trapping mechanism in follicles of lymph nodes and the animal's ability to generate an antibody response. Recent studies by Hardy, Globerson and Danon (1973) have also shown that peritoneal macrophages from 4-day old mice are incapable of reconstituting the antibody response to Shigella in irradiated adult hosts. These data clearly demonstrate that the maturity of the RES constitutes an important rate limiting factor in the ontogenic development of immunological functions.

C. Objectives in the Thesis Research

A review of present knowledge of immune ontogenesis shows that a vast amount of information has been collected on various species and strains by experiments in vivo and in vitro.

Some of the problems encountered with experimentation

in vivo are:

1) the complexity of the internal environment of the animal;
2) interference by maternal antibodies;
3) technical difficulties in reaching the fetus *in utero*; and
4) animal variability.

Factors 1, 2, and 4 make interpretation of experimental results difficult. For example, it is difficult to distinguish whether the state of immunological unresponsiveness in newborns is due to a lack of optimal conditions for functional expression of immunocompetent cells or to their immaturity per se. Experimentation on the fetus *in utero* has been carried out in some of the larger mammals and marsupials. However, unlike the laboratory rodents, these models have the disadvantages of lack of genetical uniformity, requirement for specialized handling, and high cost of the animals and their upkeep. A partial solution has been provided by the availability of tissue culture systems, but these do not allow long term studies and also the conditions *in vitro* are significantly different from those *in vivo*.

The objectives of this thesis research were (a) to develop an experimental model which would allow experimentation on the ontogeny of the immunocompetent cell *per se* with minimal influence from secondary variables such as the maturity of the RES; and (b) to employ this model in

the study of some aspects of immune ontogenesis.

CHAPTER II

DEVELOPMENT OF THE EXPERIMENTAL MODEL

Lymphocytes constitute the major component of the immune system. They not only interact with antigens in the afferent limb of the immune arc (in antigen recognition), but also participate in the efferent limb in antibody production and in cellular immunity. Although the maturation of lymphocytes during an immune response has been extensively studied, little information is available on their development, especially during early embryogenesis. A major technical problem encountered in the study of lymphocyte ontogenesis in mice is the small size of the fetuses which makes experimental work *in utero* difficult.

During early neonatal life, the immune system in mice is both anatomically and functionally poorly developed, although immunocompetent cells are present at this time (Spear *et al*. 1973). This lack of immune reactivity may be attributed to any or all of the four factors listed below:

a) the small number of lymphocytes in lymphoid tissues *in toto* ;

b) immaturity of the reticuloendothelial system (Nossal & Mitchell, 1966; Argyris, 1968);

c) the lack of opsonins or natural antibodies which help in antigen localization, processing and phagocytosis (Reade, 1968); and

d) the deficiency in lymphocyte recirculation due

to poor development of the post-capillary venules (Gowans & Knight, 1964).

Although the lymphocytes might have attained immunological competence, other environmental factors could influence the expression of immune reactivity.

To observe the ontogeny of immunocompetent cells *per se*, it is desirable to place them in an environment which will be optimal for the full expression of their immune capacity. This has been achieved in part by an *in vivo* cloning model in mice first described by Trentin and Fahlberg (1963) based on the knowledge that:

a) the lymphoid compartment of radiation-deprived mice could be reconstituted by a graft of syngeneic hemopoietic stem cells (Ford *et al.* 1966); and

b) these stem cells in the early phase of proliferation can give rise to clones of cells which may be isolated as distinct spleen colonies (Till & McCulloch, 1961).

A statistical analysis of the results of Till and McCulloch (1961) has indicated that the dose-response curve did not deviate significantly from linearity, nor was the intercept significantly different from zero. They therefore concluded that their results are compatible with the assumption that a single viable cell from the bone marrow graft gives rise to one spleen colony. Such a cell has been referred to as a colony forming unit (CFU). Thus, if cells

from a single colony were injected into another irradiated mouse, they would repopulate the recipient with the progeny of a single precursor. The formal experimental evidence for this was later presented by Becker, MuCulloch and Till (1963). Trentin and Fahlberg (1963) then designed a model to test the validity of the clonal selection theory of Burnet (1959), which postulates that only one or at the most a few, antigens should be able to elicit an antibody response from any given clone of immunocompetent cells.

In their study (Trentin & Fahlberg, 1963), lethally irradiated mice were repopulated with a small number of syngeneic bone marrow cells in order to produce separate and discrete spleen colonies. Eleven days later, cells from single, isolated spleen colonies were taken from irradiated, bone marrow repopulated primary hosts and injected into a group of lethally irradiated secondary hosts. The hemopoietic and lymphoid tissues of the secondary hosts were assumed to be entirely of donor origin as was previously shown by the chromosome analysis results obtained by Ford, Ilbery and Loutit (1957). The bone marrow and spleen cells recovered from a single secondary host 28 days later were further injected into a group of lethally irradiated tertiary hosts. Beginning on day 42 after cell injection, the surviving animals of this group were immunized sequentially with three different Salmonella preparations which showed no obvious antigenic cross-reactivity. Subsequent antibody titrations showed that these tertiary

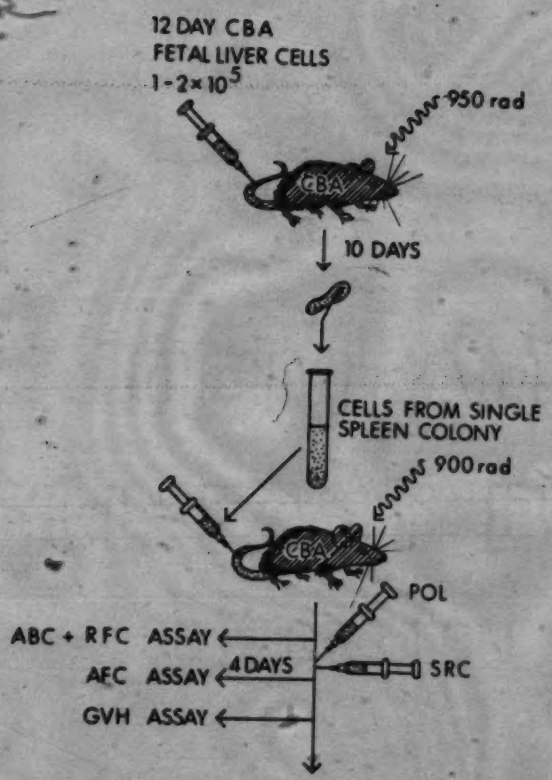

Figure 2 Experimental design for in vivo cloning of fetal liver stem cells in irradiated mice and subsequent evaluation of immunocompetence.

hosts could produce antibodies against each of the group-specific Salmonella antigens. Since the lymphoid tissue of each tertiary host presumably originated from the same bone marrow stem cell, Trentin and Fahlberg (1963) concluded that antibody forming cells were pluripotent. In a later extension of this work, Trentin *et al.* (1967) produced similar results using 4 to 13 spleen colonies to repopulate secondary hosts. Their argument against the clonal selection theory was based on the assumption that the tertiary hosts were repopulated with the cell progeny of one precursor. However they did not take into account the fact that mouse hemopoietic stem cells have self renewal properties (Siminovitch, McCulloch & Till, 1963). Thus, the cells (10.6×10^6 spleen cells PLUS 2.7×10^6 bone marrow cells) used to repopulate the tertiary hosts in their experiment should be regarded as progenies of a considerable number of stem cells.

Since immunocompetent cells do not form spleen colonies (Nekori & Feldman, 1965) the *in vivo* cloning procedure of Trentin and Fahlberg has provided a model for my study on the development of immunocompetence from a single lymphoid precursor. This model (Fig.2) adopts their method for the production of separate, discrete colonies in lethally irradiated, stem cell repopulated primary hosts. Thereafter, cells of single spleen colonies were transferred into lethally irradiated secondary hosts, and the onset of various immunologic functions followed.

As a source of stem cells, yolk sacs and livers were obtained from mouse fetuses between 9 to 12 days of gestation. Various studies (Haskill & Moore, 1970; Moore & Metcalf, 1970; Haskill, 1971) have shown that murine stem cells from yolk sac or early fetal liver have different characteristics from those in late fetal liver, spleen or adult bone marrow. It follows from their studies that the development of immunocompetence in irradiated mice repopulated with yolk sac cells should bear the closest resemblence to that occurring during normal embryogenesis. In a series of 20 experiments, 170 lethally irradiated primary recipients were repopulated with doses of yolk sac cells (from 9 to 13 day embryos) ranging from 0.1 to 1.0 x 10^6 cells. Mortality was high amongst these mice. Only 8 out of 56 primary recipients of 0.1 to 0.5 x 10^6 yolk sac cells survived 18 days after repopulation. When the repopulating dose was increased to 1 x 10^6 yolk cells, 79 out of 114 mice were still alive 14 days after repopulation. Since the primary recipients were used solely to produce spleen colonies, their long term survival was not essential. From 19 primary recipients splenectomized between days 8 to 11, a total of 10 colonies were obtained . These colonies were transferred singly into each of 10 lethally irradiated secondary recipients, all of which died within 10 days after repopulation. This suggests that yolk sac cells have few CFU when compared with bone marrow cells which have approximately 1 CFU per 10^4 cells (Till & McCulloch, 1961).

This may be due to the low spleen seeding efficiency of yolk sac cells, which has been shown by Moore and Metcalf (1970). In view of the low spleen repopulating potency of yolk sac cells, 12 day old fetal livers were used as an alternative source of stem cells.

Unlike embryos, irradiated adult mice have well organized lymphoid tissues, sufficient opsonin concentration and a mature reticuloendothelial system necessary for localization, phagocytosis and processing of antigens to enhance their immunogenicity. The development of immune reactivity in such irradiated and repopulated mice should therefore be limited only by the maturity of the cloned lymphocytes.

The following chapters describe the application of the experimental model as outlined in Fig.2 to study the development of immunological functions, including antigen recognition (antigen binding cells) and the capacity to exhibit humoral and cell-mediated responsiveness, in CBA mice.

Chapter III

GENERAL METHODS AND MATERIALS

This chapter gives an account of the materials and routine techniques used throughout the course of the study. Special methods are described separately in the pertinent chapters.

A. Mice

Mice of strains CBA/HI and Balb/c and their hybrids (CBA x Balb/c) F1 (CBF1) , were obtained from the Ellerslie Animal Farms, University of Alberta.

Male Balb/cJ, 8 to 10 weeks old, and C57Bl/6J of both sexes were obtained from Jackson Laboratories (Bar Harbour, Maine).

Animals were housed 4 to 6 per cage with free access to food and water. Irradiated recipients of cells from single spleen colonies were given subcutaneous injections of terramycin (2mg per mouse) every other day.

B. Irradiation

Mice were placed in a partitioned plexiglass circular container and received whole body irradiation at a dose rate of 114 rad per min from two Caesium^{137} sources in a 'Gamma Cell 40' (Atomic Energy of Canada Ltd., Ottawa, Ontario).

Balb/c, CBF1, and C57Bl/6J mice, used in GvH response studies were given respectively, 700, 800, and 850 rad whole

body irradiation.

C. Preparation of single cell suspensions

Cell suspensions were prepared in sterile, cold Eisen's balanced salt solution (EBSS) (Eisen et al. 1959) supplemented with 10% (v/v) fetal calf serum (FCS) (Grand Island Biological Co., Grand Island, N.Y.), 100 units of penicillin G and 100 µg of streptomycin per ml. In some experiments, Leibowitz medium (GIBCO, Grand Island, N.Y.) was used in place of EBSS.

Fetal or newborn tissues and spleen colonies were gently teased with fine forceps in either 10%FCS-EBSS or Leibowitz medium and kept on ice. Spleens from adult or perinatal mice were either forced through a double-layered nylon gauze or minced with fine scissors and then pressed through a metal sieve (pore size 45µ). The cell suspension was aspirated several times through a 23 gauge hypodermic needle to break cell aggregates. Large particles were allowed to settle out from the suspension under gravity.

Bone marrow from adult mice was obtained by flushing femur shafts with Leibowitz medium using a 26 gauge hypodermic needle. Cell aggregates were broken by aspirating the suspension several times through a 26 gauge hypodermic needle.

D. Cell Injection

Mice were placed in a 37°C incubator for 30 min before injection to dilate the tail veins. Single cell suspensions were injected through the lateral tail vein using a 26 gauge hypodermic needle.

E. Preparation of spleen colonies

Male CBA or CBF1, 7 to 9 weeks of age were used as recipients of CBA fetal liver cells.

Donors of hemopoietic stem cells were 12-day old fetuses from CBA or CBA/T6 F1 hybrids. The age of the donor was based on the presence of vaginal plugs post coitus. The day when the plug was observed was taken to be day zero of gestation.

Adult CBA or CBF1 mice were given 950 rad whole body irradiation followed a day later by an injection of 1-2 x 10^5 viable 12-day old CBA fetal liver cells in 0.1 or 0.2 ml of medium. Ten days later, these mice were killed by cervical dislocation and their spleens removed asceptically into 10%FCS-EBSS or Leibowitz medium. Only spleens with discrete colonies were used. Single colonies were dissected with fine forceps under a stereomicroscope. Each dissected colony was placed into a drop of medium in a petri dish kept on ice. A single cell suspension was prepared from each colony in approximately 0.4 to 0.7 ml of medium. In some experiments, the cell suspension was prepared from pooled

spleen colonies.

F. Cell count

Cell counts were done in a hemocytometer and cell viability determined by the eosin dye exclusion test (Hanks & Wallace, 1950).

G. Thymectomy

Thymectomy was performed in neonates within 24 Hr of birth or in 15-day old animals using the method of Miller (1960, 1961). Completeness of thymectomy was confirmed by gross and microscopic examination of the mediastinal tissue. Only results from completely thymectomized animals are reported. Thymectomized animals were used when they were 8 to 12 weeks old.

H. Antigens

Flagellar H-antigen was prepared from Salmonella adelaide (strain 1338, H-antigen, f,g; O-antigen, 35) by the method of Ada et al. (1964). The polymerized form, POL, was used for immunization.

Sheep erythrocytes (SRC) were collected into Alsever's solution and kept at 4°C. Before use, they were washed 3 times in Dulbecco's phosphate buffered saline (PBS), pH 7.4.

I. Immunization

For immunization, each mouse was injected intraperitoneally with 25 µg of POL in 0.25 ml PBS followed by 0.1 ml of a 10% (v/v) suspension of washed SRC (approximately 10^8 cells) given via the same route.

J. Enumeration of antibody forming cells

Antibody forming cells (AFC) to POL were assayed by the adherence colony method of Diener (1968), and AFC to SRC were measured by the method of Cunningham and Szenberg (1968).

K. Statistical analysis of data

Numerical data in groups were expressed as their mean ± one standard error of the mean (S.E.). Comparisons were made using p-values calculated by the student's t-test. Significance was accepted at $p<0.05$.

CHAPTER IV

ONTOGENY OF HUMORAL RESPONSIVENESS

A. Introduction

Experimental determination of the ontogeny of immunocompetent cells in mice has been much impeded by the technical difficulty of gaining access to the early fetus *in utero*. This problem may be overcome using the *in vivo* cloning model as outlined in Fig.2(p.35). Using this, a study was done on the time course of antigen recognition in a population of lymphocytes originating from a single or at the most a few stem cells.

It is now well established that immune recognition involves the specific binding of antigens onto immunoglobulin-like receptors on the surface of immunocompetent cells (Byrt and Ada, 1969). These cells are referred to as antigen binding cells (ABC). The ontogenic development of antigen recognition can be determined by following the sequential appearance of ABC for different antigens. A simple method for detecting ABC for a soluble protein antigen was first described by Naor and Sulitzeanu (1967) using ^{125}I-labeled BSA followed by autoradiography. This method allows the visualization of ABC and also the assessment of their frequency in a lymphocyte population. However, the low density of surface receptors on T-cells precludes their detection by this assay (Nossal *et al.*

1972). For particulate antigens like xenogeneic erythrocytes, ABC can be identified as cells surrounded by a corona of erythrocytes (Zaalberg, 1964; McConnell et al. 1969). Because of their appearance, such ABC are termed rosette forming cells (RFC). The RFC technique has been shown to detect ABC of both B and T cell lineages (Bach & Dardenne, 1972).

Extensive studies have been directed towards the immunologic functions of ABC. ABC have been shown to be essential for immune responsiveness as removal of specific ABC by 'radiation suicide' using ^{131}I-labeled antigen (Byrt and Ada, 1969) or by passage through an antigen-coated column (Wigzell and Makela, 1970) results in the abolition of a specific humoral response. Using a flourescence-activated cell sorter, Julius, Masuda and Herzenberg (1972) have provided direct evidence that antigen binding cells are precursors of antibody producing cells. These studies are concerned with antigen binding B-cells. It has been observed that light chains are present on T-cells (Bankhurst, Warner & Sprent, 1971), and probably form part of the antigen binding receptors. Evidence for the immunologic functions of antigen binding T-cells is derived from the abrogation of the T-cell mediated GvH response (Mason & Warner, 1970) and the hapten-carrier response (Cheers et al. 1971) following anti-immunoglobulin treatment.

A lymphocyte, at the time of acquisition of its antigen

binding capacity during ontogeny may not be able to participate in an immune response upon antigenic challenge. Therefore it would be of interest to find out if antigen recognition and immune responsiveness develop simultaneously.

The aim of the following experiments is to establish the time of onset of recognition and humoral responsiveness to Salmonella flagellin and SRC in recipients of single spleen colonies derived from fetal liver stem cells.

B. Methods

1. Chromosome Analysis

Since the T6 chromosome can only be visualized in cells at metaphase, each test animal received 150 µg of colcemide (GIBCO, Grand Island, N.Y.) intraperitoneally 3 hr prior to assay. This was to arrest cell division at metaphase. The animals were killed by cervical dislocation and cells were obtained from the femur and/or the spleen. Chromosome preparations were carried out by a modification of the method of Fox and Zeiss (1961) and stained with Giemsa.

2. Preparation of Radioiodine-labeled antigen (^{125}I-MON)

Polymerized flagellin, POL, was converted into the monomeric form, MON, just before iodination by mixing with 1/20th volume of 1N HCl at room temperature for 15 min, followed by neutralization with an equal volume of 1N NaOH. Iodination was performed using the chloramine-T method of

Greenwood, Hunter and Glover (1963). One hundred µg of MON in 0.1 ml were added to 10 mCi (in 30-35 µl) of carrier-free, radioactive iodine ^{125}I (New England Nuclear, Boston, Mass.), followed by 500 µg of chloramine-T (1% solution in PBS). The mixture was allowed to react at room temperature for 20 min and the reaction was terminated by adding 700 µg of $Na_2S_2O_5$ (1% solution in PBS). The labeled antigen was separated from free iodide by chromatography on a small (6 x 60 mm) column of Sephadex G-25 (Pharmacia, Uppsala) which was pretreated with BSA. The specific activity of different preparations of labeled antigen was 40-90 µCi/µg of protein.

3. Assay for Antigen Binding Cells (ABC) to ^{125}I-labeled MON

The precedure used to enumerate ABC was that of Byrt and Ada (1969), except that Dulbecco's minimal essential medium (MEM) was replaced with 10%FCS-EBSS. Before incubation with the labeled antigen, the cells were spun through 100% FCS to remove debris. In all instances, the amount of antigen used ranged from 500 to 750 ng protein in 0.2 ml of medium containing sodium azide at a final concentration of 1.5×10^{-2} M. From 2 to 10 x 10^6 cells were incubated with the labeled antigen in an ice bath for 30 min. Thereafter, the cells were washed 3 times in EBSS. The pellet was resuspended in 100% FCS and the cells smeared onto gelatin coated slides. These were fixed in methanol-acetic acid-water mixture (89:1:10) for 30 min and

subsequently washed for 30 min in running water and then processed for autoradiography. Owing to the presence of azide, no phagocytic cells were found to be labeled in any experiment.

4. Autoradiography and Scoring for ^{125}I-MON Binding Cells

The slides with cell smears were dipped in Kodak NTB-2 photographic emulsion and dried. They were stored for 2 to 4 days in the dark at 4°C in the presence of a drying agent (anhydrous calcium sulphate), developed in Kodak developer D-19, fixed and stained with Giemsa. A cell was considered labeled when the number of grains associated with it was at least 20 times above background. Following this criterion, the error probability of scoring a labeled cell was less than 0.01%. In cases of heavily labeled cells, the grains in the photographic emulsion were dissolved to reveal the underlying cell. Only intact cells were accepted as positive.

5. Rosette Forming Cell (RFC) Technique

This assay was carried out according to the method of Wilson (1971). A mixture of 5 x 10^6 viable cells and 5 x 10^6 SRC (0.08% suspension in 10%FCS-EBSS) in 1 ml were spun together for rosette formation. The pellet was allowed to stand on ice for 5 min and was then resuspended. The number of rosette forming cells (RFC) was counted in a hemocytometer. Only complete rosettes were scored. Two determinations were made for each sample. The total number

TABLE 2 Chromosome analysis of spleen and bone marrow cells of secondary recipients

Animal No.[1]	Days after transfer of cloned cells	Cell source	Cells with donor T_6 chromosome / Total cells scored
1	20	spleen	57/62
2	30	spleen	22/23
		bone marrow	25/26
3	30	bone marrow	6/6
4	30	bone marrow	39/42
5	30	bone marrow	17/17

1 Single animals from five different experimental groups were tested

of lymphocytes screened ranged from 9,000 to 20,000.

C. Results

1. Cellular Identity of Hemopoietic Tissue in Recipients of Cloned Cells

In experiments involving the hemopoietic reconstitution of irradiated animals, there is the possibility that the survival of recipients may be due to endogenous repopulation rather than to reconstitution by the injected cells. Therefore, the origin of dividing cells in the hemopoietic tissue of spleen colony recipients was determined. For this purpose, donor cells were obtained from fetal livers of CBA/T6 hybrids which carry the T6 chromosome marker (Carter, Lyon & Phillips, 1955) identifiable in karyotype analysis. Fetal liver cells from CBA/T6 hybrids were allowed to proliferate for a period of 10 days in irradiated CBA primary recipients. Thereafter, single spleen colonies were transferred into another group of irradiated CBA recipients. Chromosome analysis was carried out as described on spleen colony recipients 20 and 30 days following cell transfer.

Table 2 shows the results obtained from 5 animals, each taken from a different experimental group. More than 92% of the metaphase figures examined revealed the T6 donor cell marker. This high proportion of donor cells renders it inconsistent with extensive endogenous repopulation. Furthermore, all of 40 control mice which were irradiated with 900 to 950 rad but not given cells, failed to show

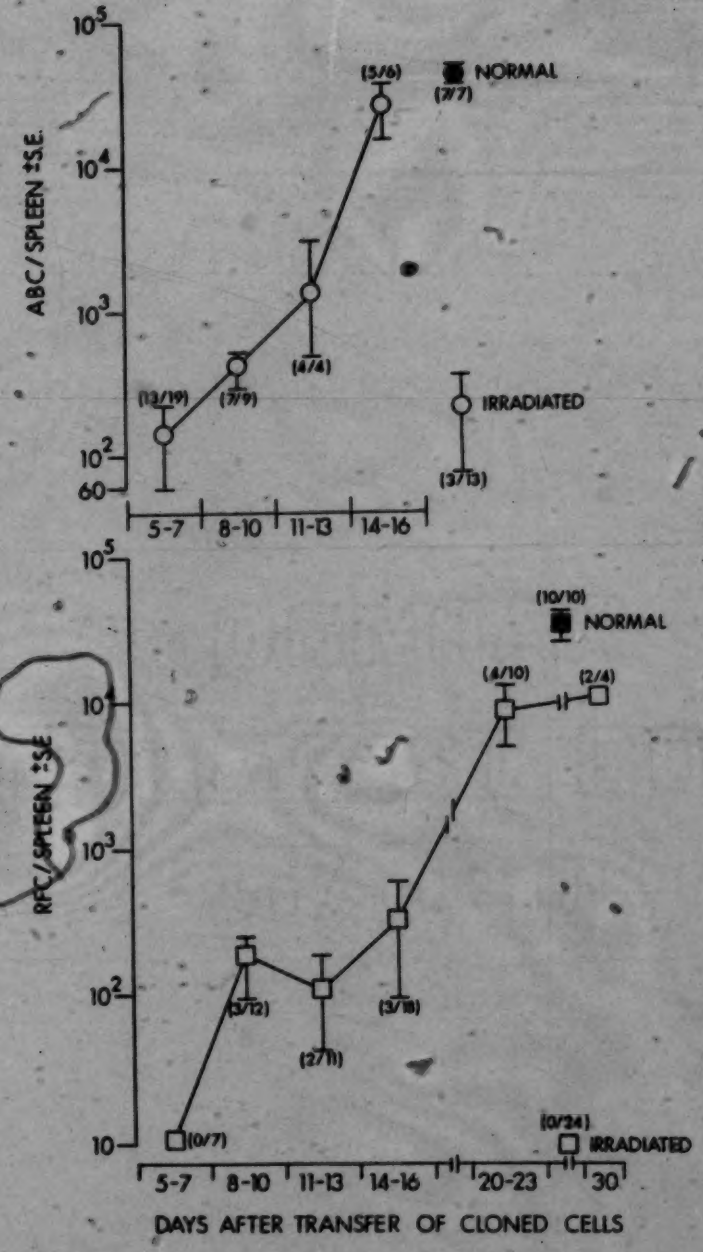

Figure 3 Development of antigen recognition in recipients of cloned cells.
The ratio of mice with splenic ABC (o-o) or RFC (□-□) to the total number tested per group is within parentheses. Irradiated control mice were exposed to 900-950 rad but not given cells. They were killed on days 3 to 15 post-irradiation and their spleen cells assayed for ABC or RFC in the same way as for experimental animals.

spleen colonies within a period of 8 to 15 days after irradiation. It was therefore concluded that lymphoid cells of irradiated mice repopulated with single spleen colonies are of donor origin.

2. Development of Antigen Recognition

a. Antigen recognition capability of spleen colonies

To determine whether or not 10-day old spleen colony cells can recognize antigens, cells pooled from several spleen colonies on the day of transfer were assayed for their ability to bind ^{125}I-MON and SRC. The results show that less than one ABC per 100,000 or one RFC per 20,000 lymphocytes was detectable. This confirmed the absence of antigen binding cells to these antigens in the spleen colonies at this time of clonal development.

b. Antigen recognition in recipients of single spleen colonies

Individual spleens from lethally irradiated recipients of single spleen colonies were tested for their contents of ABC to ^{125}I-MON or RFC at various times after repopulation. The results are shown in Fig. 3.

Starting from 5 to 7 days after the transfer of such colonies, the number of ^{125}I-MON binding cells increased exponentially with time with a correlation coefficient (r) of 0.9929. By days 8-10, the number of ABC was already significantly higher when compared with that of the previous time point ($p<0.01$). Moreover, throughout the observation

period, ABC were detectable in more than half of the mice in each group. Taking into account that the cells had resided for 10 days in the primary recipients prior to transfer, it was concluded that the ability of the immune system to recognize Salmonella flagellin antigen was established between 18-20 days of development from a stem cell.

In contrast to results obtained with ABC, RFC were first detected between day 8-10 in spleen colony recipients and remained at the same level throughout the first 13 days of observation. An increase in the level of RFC was, however, observed between days 14-16 following single colony transfer. Furthermore, it was observed that the proportion of experimental mice with detectable splenic RFC was low throughout this period. By days 20-23, a sharp increase of RFC had occurred, reaching 30% of the normal level, and 4 out of 10 mice tested were positive. Similar numbers of RFC were found in 2 out of 4 mice tested on day 30 while none was detected in the remaining two. Therefore it appears that the competence for SRC recognition required a total of 30-33 days to develop from a single stem cell.

c. Rosette forming cells in recipients of pooled spleen colonies

The number of RFC was assayed in irradiated mice repopulated with cells pooled from an average of 4-5 colonies to find out if an increase in the number of lymphoid precursors could result in earlier appearance of

TABLE 3 Rosette forming cells (RFC) in recipients of pooled spleen colonies[1]

Days after cell transfer	No. of mice with RFC in spleen / Total No. tested	RFC/spleen in positive mice
6	0/4	-
8	0/4	-
12	0/4	-
15	0/4	-
20	2/4	10,765 21,904

1 CBA mice were irradiated (900 rad) and repopulated with 4 - 10 x 10^6 spleen colony cells. At different times thereafter, groups of 4 mice were killed and the number of RFC in each spleen was assayed.

RFC. At various times after the transfer of pooled spleen colony cells, individual spleens of these recipients were assayed for their contents of RFC and the results are shown in Table 3.

As in the case of single spleen colony recipients, no RFC were detectable in the spleens of irradiated mice reconstituted with pooled colonies up to 15 days after cell transfer. On day 20 after cell injection, 2 out of 4 mice tested were positive for spleen RFC.

3. Development of Humoral Responsiveness to POL and SRC in Recipients of Single Spleen Colonies

To determine the development of humoral responsiveness, groups of irradiated recipients of single spleen colonies were immunized with POL and SRC at various times following repopulation. Four days after immunization, these animals were killed and individual spleens were assayed for the number of antibody forming cells (AFC) to either antigen. The results are shown in Fig. 4.

An exponential increase of AFC to POL was observed starting with animals immunized 0-9 days after colony transfer. By days 10-19, the level of AFC to POL detected was already comparable to that in normal control mice. This suggests that humoral immune responsiveness to the flagellar antigen was established between 20-29 days of development from a single stem cell.

On the other hand, no appreciable response to SRC was

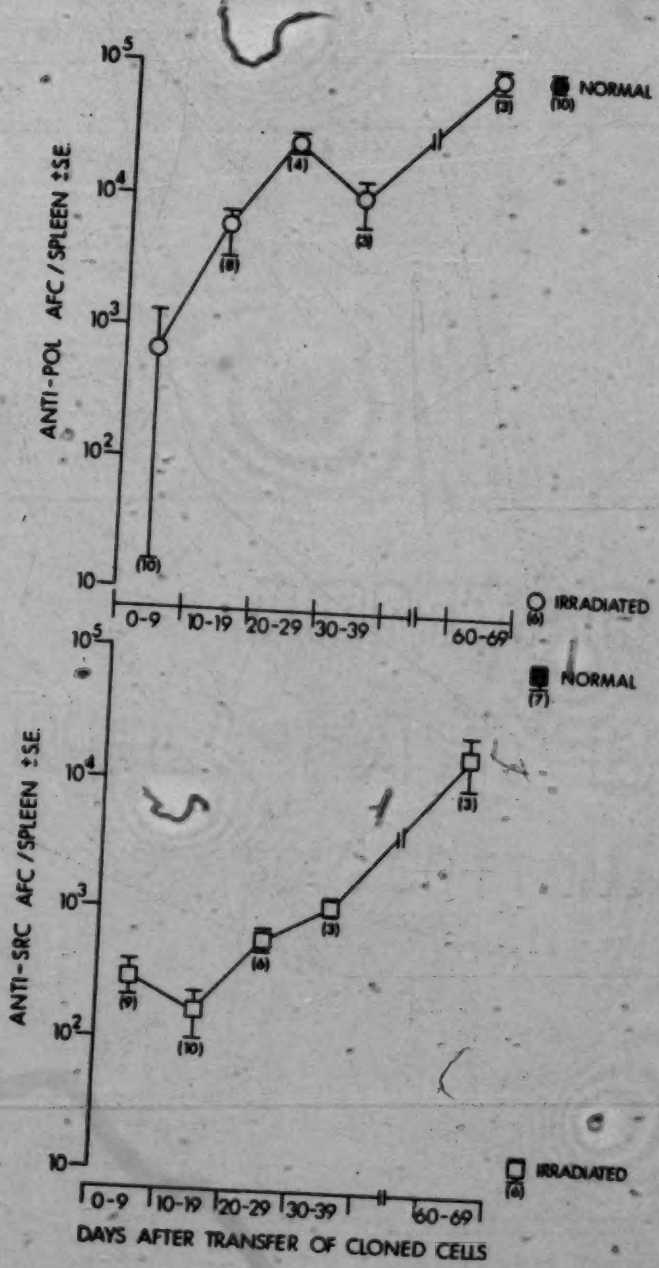
ANTI-POL AFC / SPLEEN ±SE
10^5
10^4
10^3
10^2
10
NORMAL
IRRADIATED
0-9
10-19
20-29
30-39
60-69
ANTI-SRC AFC / SPLEEN ±SE
DAYS AFTER TRANSFER OF CLONED CELLS

Figure 4 Development of humoral immune responsiveness in recipients of cloned cells.
The number of mice per group is within parentheses. All mice tested were found to have AFC to POL or to SRC with the exception of a) group (0-9) in which only 2 out of 10 mice were positive for AFC to POL; and b) group (10-19) in which the ratios of positive/total mice tested for AFC to POL or to SRC were 7/8 and 7/10 respectively. Irradiated control mice were exposed to 900-950 rad but not given cells. They were immunized on days 8 to 15 post-irradiation and assayed for AFC 4 days later.
(a) Anti-POL AFC (o-o)
(b) Anti-SRC AFC (□-□)

observed up to day 19 after colony transfer. By day 20-29, the number of AFC to SRC had reached a significantly higher level than was observed between day 0-9 ($p<0.001$). From then on, a steady increase of responsiveness was observed up to 65 days after colony transfer. At this time the magnitude of the response to SRC had reached 85.4% of that of normal control values. It was concluded that under the present experimental conditions, the capacity of the immune system to produce antibodies to SRC was not detectable before 30 days of development from a single stem cell.

D. Discussion

The experimental model used in this study is based on the observation that a single hemopoietic spleen colony of the mouse, when transferred to a lethally irradiated syngeneic recipient, may restore its lymphopoietic system. Although the long term survival expectancy of such recipients is only 20%, studies using chromosome markers showed that such survival was indeed due to the restoration by donor cells of the blood forming tissue.

The study described in this chapter addresses itself to the sequential expression of antigen recognition and humoral immune responsiveness during ontogeny. For polymerized flagellin, a period of 18 to 20 days of clonal expansion from a fetal stem cell was required for the establishment of the antigen recognition potential. This figure compares favourably with the 20 days estimate by Jerne (1970) for the

complete generation of antigen recognition diversity in a lymphocyte population derived from a single precursor cell, on the assumption that clonal diversity of antigen recognition results from somatic mutation.

Next, the onset of antigen recognition and that of humoral immune responsiveness in recipients repopulated with single spleen colonies was compared. The data show that:

a) Antigen recognition developed before immune responsiveness.

b) Recognition and humoral immune responsiveness against a T-independent antigen, POL (Diener, O'Callaghan & Kraft, 1971), were expressed earlier than those against SRC, a T-dependent antigen (Miller & Mitchell, 1967).

The results on the appearance of flagellin-binding cells are compatible with findings of Nossal and Pike (1972) who showed that the number of immunoglobulin bearing B-cells increases rapidly in the spleen of the CBA fetus during the period between 16 days of gestation and birth. This was confirmed by Spear et al. (1973) in Swiss L mice. The present data show that a period of 33 days of development was required before a significant level (30% of normal) of RFC was detectable. This agrees remarkably well with the data of Bach et al. (1971) who showed that in mice, RFC to SRC did not appear until 7 to 14 days after birth, that is after a total of 26 to 33 days of development from conception. However, Spear et al. (1973) could detect a low

level of RFC to SRC in the spleens of fetal mice at 16 days of gestation. Since >50% of RFC in the spleen have been shown to be θ-positive T-cells (Greaves and Moller, 1970; Bach & Dardenne, 1972), the discrepancy of results from different laboratories may reflect a strain variation in T-cell maturation rate.

The data show that antigenic recognition of SRC developed later than that of POL. One possible explanation for this discrepancy is the difference in sensitivity of the two assays. Bankhurst and Wilson (1971) have demonstrated that in the case of chicken globulin, more ABC were detected by using ^{125}I-labeled chicken globulin combined with autoradiography than by rosette formation with chicken globulin-coated SRC. Similar observations have been made by Langman (1972) with respect to POL. These authors proposed that there is probably a spectrum of affinity among antigen binding cells and that only cells with the most avid receptors form rosettes. Thus the late onset of SRC recognition may reflect a slow increase of high affinity ABC during lymphoid maturation. In view of the findings that a large proportion of RFC is comprised of T-cells (Greaves and Moller, 1970; Greaves and Hogg, 1971; Bach & Dardenne, 1972), another explanation for the late appearance of SRC-binding cells is that T-cells develop slower than B-cells. Since only the spleen was examined, there is also the possibility of SRC-binding T-cells in the thymus waiting to emigrate into peripheral organs. Consistent with this is

the finding of Fidler (1972) which shows that immunocompetent T-cells appear first in the thymus and then in the spleen.

There is now evidence that in the adult mouse, cells capable of binding ^{125}I-labeled antigen, as detected by conventional autoradiography, are B-cells (Nossal et al. 1972) and that ABC are precursors of antibody forming cells (Julius, Masuda & Herzenberg, 1972). Therefore it is expected that the increase in the number of ABC during ontogeny of the immune system should parallel the emergence of humoral responsiveness. This is so in the case of POL, a T-independent antigen, although humoral responsiveness to this antigen lagged somewhat behind the manifestation of antigen recognition. This may be accountable to a limited number of immunocompetent cells present early in development.

The results show a sequential development of responsiveness, first to POL and then to SRC, similar to the findings of Silverstein et al. (1963) in fetal lambs. This may be due to the fact that humoral responsiveness to SRC is T-dependent (Miller & Mitchell, 1967). Evidence in support of this view has been provided by several groups (Chiscon & Golub, 1972; Arrenbrecht, 1973; Spear & Edelman, 1974).

Apart from T- and B-cells, a third cell type - the adherent (A) cell - is essential for immune responsiveness to SRC (Mosier, 1967; Shortman et al. 1970), but not to POL (Diener, Shortman & Russell, 1970). Therefore, the onset of

humoral responsiveness to SRC in irradiated recipients of cloned cells may be limited by the maturity of A cells. Argyris (1968) has shown that the response to SRC in young mice at least 3 days after birth can be restored by injection of adult peritoneal macrophages. Recent studies of Hardy, Globerson and Danon (1973) have provided data in accordance with the idea that peritoneal macorphages in mice during the first 4 days after birth are immature and incompetent to participate in immune reactions. Contradictory to these, Fidler, Chiscon and Golub (1972) reported that spleen adherent cells from newborn mice could co-operate with lymphocytes in the SRC response in vitro . This controversy cannot be reconciled until the relevance of the in vitro activity of adherent cells (Mosier, 1967) to the role of macrophages in the antibody response in vivo is clarified.

Finally, it is equally likely that the lack of humoral responsiveness to SRC in irradiated and repopulated mice is due to the immaturity of both T- and A cells.

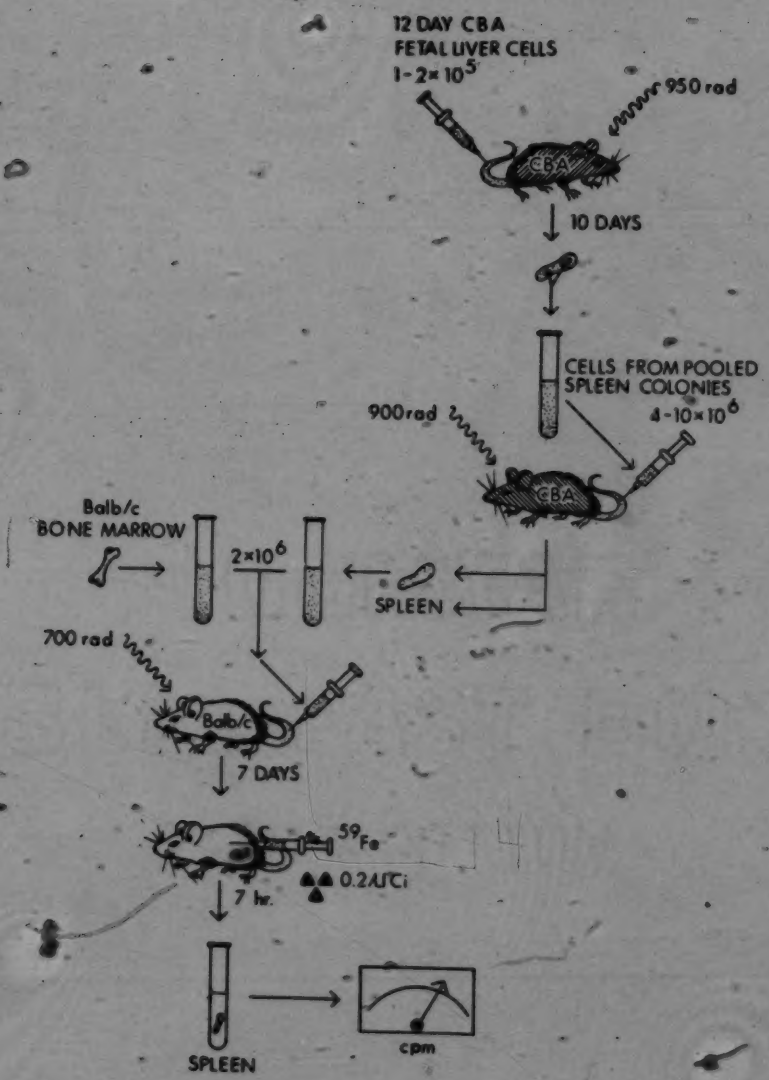

Figure 5 Experimental protocol for the study of the development of GvH reactivity in recipients of cloned cells.

CHAPTER V

ONTOGENY OF CELL-MEDIATED IMMUNITY

A. Introduction

In cell mediated immunity, antigenic stimulation leads to the generation of activated lymphocytes which exert their cytotoxic effects upon direct contact with the target cell. This state of immunity can only be adoptively transferred by immuned cells. In the following study the ontogeny of cell-mediated immunity in irradiated mice repopulated with cloned cells was followed according to the protocol outlined in Fig.5. GvH reactivity was taken as a criterion to assess the degrees of immunocompetence.

A GvH reaction occurs when an immunologically incompetent host receives an allograft containing immunocompetent lymphocytes. Therefore the ability to elicit symptoms of a GvH reaction in appropriate hosts is an indication of the state of maturity of lymphoid cells per se, thus excluding the influence of extrinsic factors such as the maturity of the RES.

The Simonsen splenomegaly assay (1957) is commonly used to assess GvH reactivity. This method is based on the fact that mature lymphoid cells when injected into allogeneic or semiallogeneic neonatal mice or chickens cause cellular proliferation and thus spleen enlargement. GvH reactivity is expressed as a spleen index which is computed from the

spleen to body weight ratio of experimental animals and littermate controls. A linear relationship exists between the spleen index and the logarithm of the dose of lymphocytes injected. Recently, Blomgren and Andersson (1972) have devised an alternative method for the assay of GvH reactivity. They observed that ^{59}Fe uptake in the spleens of irradiated mice which were repopulated with syngeneic bone marrow could be suppressed by the presence of allogeneic lymphoid cells in the inoculum. Furthermore, the degree of suppression of erythroid cell growth was found to be directly proportional to the dose of allogeneic cells, and offers a quantitative assay for the reactivity of the grafted cells. Thus this method was adopted for the present study because it requires fewer cells than the Simonsen assay.

B. Methods

1. Assay for Graft versus Host Reactivity and Evaluation of Results

Male, 8-12 weeks old Balb/c mice were given 700 rad irradiation and within 24 hr, groups of 5 or 6 mice were each injected intravenously with 2×10^6 Balb/c bone marrow cells alone, or together with varying doses of allogeneic spleen cells.

Seven days after cell injection, each mouse was injected intraperitoneally with 0.2 μCi of ^{59}Fe in the form of ferrous citrate (New England Nuclear, Boston, Mass.).

The animals were killed seven hours later, their spleens were removed, and fixed in Bouin solution. The radioactivity of each spleen in cpm was measured in a well-typed scintillation counter (Packard Tricarb Scintillation Spectrometer, Model 3002, Downers Grove, Il.). The extent of ^{59}Fe uptake of each spleen was expressed as the percentage of the total amount of radioactivity injected. This was necessary as the specific activities of the different batches of ^{59}Fe used varied from 9.8 to 32.6 mCi/mg.

Each experiment included 2 control groups:

a) a bone marrow control group in which each Balb/c host was given 2×10^6 syngeneic bone marrow cells alone; the mean percent of ^{59}Fe uptake in this group was taken to be 100% for the determination of the degree of suppression by allogeneic cells using the following formula:

$$\% \text{ suppression} = \left(1 - \frac{\text{mean } \%^{59}Fe \text{ uptake of test gp}}{\text{mean } \%^{59}Fe \text{ uptake of b.m.control gp}}\right) \times 100\%$$

b) a normal control group in which each Balb/c host was given 2×10^6 normal CBA spleen cells in addition to 2×10^6 Balb/c bone marrow cells; the suppression of ^{59}Fe uptake in this group was used as the maximum or 100%

TABLE 4 Erythropoiesis in irradiated and repopulated Balb/c mice[1]

Treatment	Number of mice	Mean% ^{59}Fe uptake ± S.E.
700 rad	12	0.8 ± 0.1
700 rad and 2×10^6 Balb/c bone marrow cells	12	13.5 ± 0.5

1 Erythropoiesis was measured in terms of % ^{59}Fe uptake in the spleen. Balb/c mice were exposed to 700 rad irradiation alone or followed by 2×10^6 Balb/c bone marrow cells given intravenously. 7 days later, each mouse was injected with 0.2 μCi ^{59}Fe in 0.3 ml PBS intraperitoneally 7 hr before it was killed.

normalized activity for quantitative comparison of the activity of the test lymphocytes.

The p-values were calculated by the student's t-test using % ^{59}Fe uptake as a parameter.

C. Results

1. Erythropoiesis in Irradiated and Repopulated Balb/c Mice

Balb/c mice exposed to 700 rad irradiation were used as hosts in all experiments. The extent of erythroid cell proliferation in the host animals was assayed by a 7 hr ^{59}Fe uptake 7 days after cell injection. As 700 rad was not a lethal dose of irradiation for Balb/c mice, there could have been endogenous erythroid cell proliferation in the recipients. To test this, 24 Balb/c mice were irradiated and 12 of them were each injected with 2×10^6 syngeneic bone marrow, the other 12 were left uninjected. Seven days later, each of the 24 mice was given 0.2 μCi of ^{59}Fe intraperitoneally and their splenic incorporation of the isotope assayed. As shown in Table 4, mice irradiated and repopulated showed an average ^{59}Fe uptake of 13.5% in their spleens whereas mice irradiated only showed an average ^{59}Fe uptake of only 0.8%. Thus hemopoietic activity of repopulated mice is 17 times that of unrepopulated mice. Therefore endogenous erythroid cell proliferation should have little effect on the results.

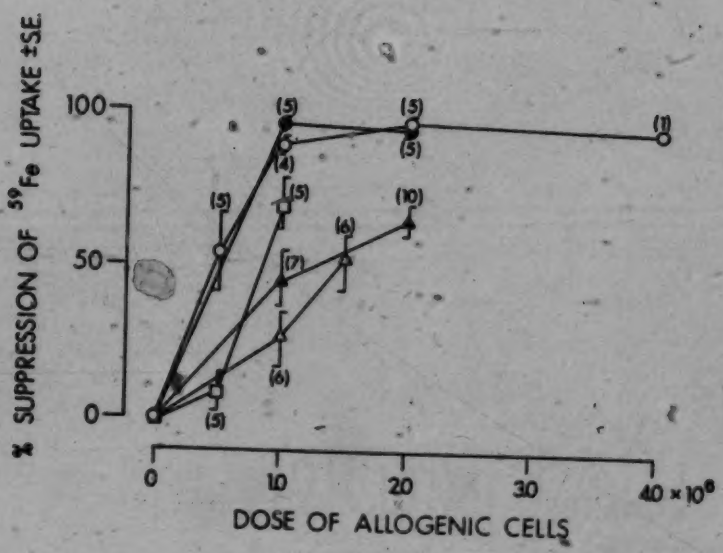

Figure 6 Cell dose-response curve of GvH reactivity in normal CBA spleen cells.
GvH reactivity was assayed by the suppression of erythropoiesis as measured by ^{59}Fe uptake of Balb/c bone marrow cells in irradiated Balb/c hosts. Each irradiated Balb/c mouse was injected with 2 x 10^6 syngeneic bone marrow cells alone or with CBA spleen cells. Seven days later, 0.2 µCi of ^{59}Fe were injected into each test animal which was killed 7 hr later and its spleen ^{59}Fe uptake determined. The data are from 5 experiments (each represented by a different symbol) performed under identical conditions. The number of mice per group is within parentheses. There were 5 mice in the group not given allogeneic cells in each experiment. Originally there were 5 mice in the group given 4 x 10^6 allogeneic cells in experiment (o), but 4 of them died of severe GvH disease by the time of ^{59}Fe injection.

2. Cell Dose-Response Relationship of GvH Activity in Normal CBA Spleens

To determine the optimal dose of CBA splenic lymphocytes to be used in these studies, a cell dose-response curve was constructed as shown in Fig.6. As the dose of CBA spleen cells was increased from 0.5 to 2 x 10^6, a progressive decrease in splenic ^{59}Fe incorporation in Balb/c hosts was observed. However, the suppressive effect produced by 0.5 to 1.5 x 10^6 CBA spleen cells varied between experiments. When 2 x 10^6 cells were used, more consistent results were obtained with a suppression of Balb/c erythroid cell growth ranging from 60 to 98%. A further doubling of this cell dose did not increase the suppressive effect on Balb/c erythroid cell growth although a more severe GvH disease was produced and most animals were dead by day 7. Therefore 2 x 10^6 CBA spleen cells were chosen as the standard dose of allogeneic cells in this study. Although the degree of suppression produced by spleen cells could not be quantitated as well as reported by Blomgren and Andersson (1972) who used normal or cortisone resistant thymocytes, the assay did serve as an indication of the relative content of immunocompetent cells in the splenic lymphocyte population from the different donors.

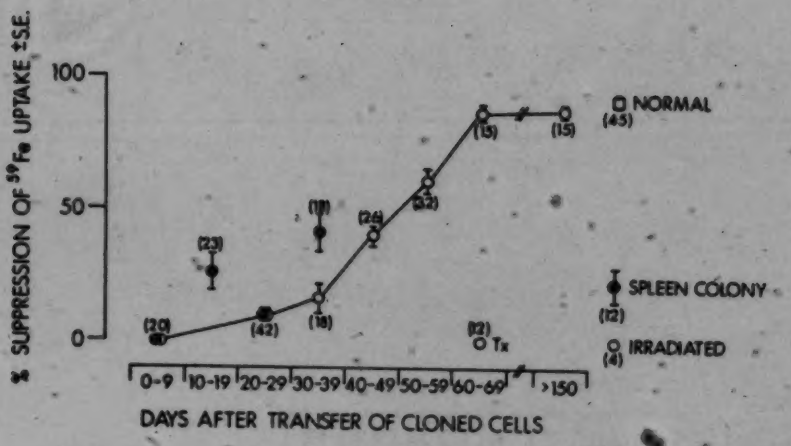

Figure 7 Development of GvH reactivity in CBA recipients of cloned cells.
At the time of assay, 2 x 10^6 (o-o) or 5 x 10^6 (•-•) spleen cells from each CBA donor were injected with 2 x 10^6 Balb/c bone marrow cells into each of 5 or 6 irradiated (700 rad) Balb/c test mice. Seven days later 0.2 µCi ^{59}Fe were injected into each Balb/c host. The test animals were killed 7 hr later and the spleen ^{59}Fe uptake determined. The number of Balb/c test mice per group is within parentheses. Irradiated control CBA mice were exposed to 900 rad but not given cells. They were killed on day 8 post-irradiation and their spleen cells were assayed for GvH reactivity.

• Results using 2 x 10^6 or 5 x 10^6 spleen cells were not significantly different from each other and were pooled.

Tx Thymectomized (15 days old) CBA recipients of cloned cells.

3. Development of GvH Reactivity in Spleen Colony Recipients

In order to follow the development of immunological competence over an extended period, the high mortality of animals repopulated with single colonies was avoided by using several colonies per recipient. This change in the experimental procedure was adopted since it was shown in Chapter IV (Table 3) that the patterns of development of SRC recognition between animals repopulated with single or multiple (4 to 5) spleen colonies were similar. The use of irradiated mice as '*in vivo* incubators' for the developing lymphoid cells made it necessary to determine whether or not the spleens of lethally irradiated CBA mice contained residual radiation resistant cells capable of participating in a GvH response. Thus controls were included to test the residual GvH reactivity in CBA mice exposed to 900 rad irradiation 8 days previously. To study the ontogeny of GvH reactivity in recipients of cloned cells, groups of spleen colony recipients were killed at approximately 10-day intervals following colony transfer. Each individual spleen was assayed for its GvH reactivity in 5 or 6 irradiated Balb/c hosts and the results are summarized in Table 5 and Fig.7.

Cloned fetal liver cells from the spleens of irradiated recipients in the first 9 days after cell transfer showed no GvH activity when cell doses of 2 or 5 x 10^6 cells were used. Between 10-19 days of maturation, 5 x 10^6 spleen

TABLE 5 Development of graft versus host (GvH) activity[1] in irradiated CBA recipients of cloned cells[2]

Days after transfer of cloned cells	Dose of CBA spleen cells (x 10^6)	No. of mice tested[3]	No. of mice with positive GvH activity	p-values[4] of GvH positive mice
0 - 9	2 or 5	4	0	
10 - 19	5	4	0	
20 - 29	2 or 5	7	0	
30 - 39	2	3	1	<0.05
	5	3	3	<0.05
				<0.005
				<0.001
40 - 49	2	5	3	<0.05
				<0.005
				<0.001
50 - 59	2	6	6	<0.05
				<0.005 in 4 mice
				<0.001
60 - 69	2	3	3	<0.001 in all 3 mice
150	2	3	3	<0.005
				<0.001
				<0.001

1 GvH activity was measured as the extent of suppression of ^{59}Fe uptake by allogeneic (CBA) spleen cells in irradiated (700 rad), bone marrow (2 x 10^6 cells) repopulated Balb/c hosts.

2 Each CBA recipient was given 4 - 10 x 10^6 cells from pooled 10-day old spleen colonies derived from syngeneic fetal liver stem cells.

3 From each repopulated CBA mouse 2 or 5 x 10^6 spleen cells were injected into each of 5 or 6 Balb/c test mice.

4 p-values were determined by the student's t-test on comparisons made between % ^{59}Fe uptake in test groups and the bone marrow control group in which irradiated Balb/c test mice received bone marrow cells alone.

cells showed an insignificant GvH activity equivalent to a mean suppression of 26%. However, by days 20-29, 2 or 5 x 10^6 cells could only produce a mean GvH activity of 9.4% suppression. Thereafter, a progressive increase in GvH activity was observed in the splenic lymphocyte population of the recipients of cloned fetal liver cells. By days 30-39, 2 x 10^6 spleen cells showed a mean suppression of 16% and 5 x 10^6 cells were able to produce a significant ($p<0.02$) suppression with a mean of 41.4%. From this time onwards, a rapid and linear increase of GvH activity was observed reaching a level comparable to that exhibited by normal CBA spleen cells by days 60-69.

Table 5 shows the number of recipients of cloned fetal liver cells with significant ($p<0.05$) GvH activity in each of the groups tested at various times following repopulation. None of the animals tested before day 30 showed significant GvH activity. Beginning from days 30-39, the proportion of GvH positive spleens increased with time. It was thus concluded that cell-mediated immunity in recipients of cloned cells is established between 40-49 days of lymphoid development starting from a small number of fetal liver stem cells.

4. Influence of the Thymus in the Development of GvH Reactivity

Thymic influence on the development of cell-mediated immunity was confirmed in this study since no GvH activity

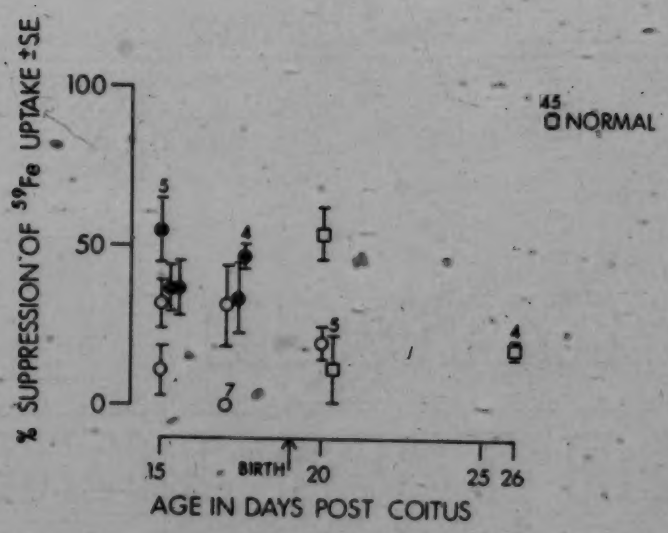

Figure 8 Development of GvH reactivity in fetal and perinatal CBA mice.
At the time of assay, 2 x 10^6 liver (o-o) or spleen (□-□) or 5 x 10^6 liver (•-•) cells prepared from a pool of mice were tested for their GvH activity as described in legend of Figure 6. There were 6 Balb/c test mice in each group unless indicated otherwise. Results from normal and irradiated control CBA mice were taken from Figure 7.

was detected in thymectomised (at 15 days of age) recipients of cloned cells assayed 65 days after repopulation (Fig.7). By this time of development in normal irradiated recipients, GvH reactivity was established.

5. GvH Activity in Fetal and Perinatal CBA Mice

In order to establish if the results obtained from this experimental model were compatible with those obtained during normal development, the ontogeny of GvH activity in fetal and perinatal CBA mice was followed from 15 days of gestation to 7 days post partum. The results (Fig.8) showed a wide variation in GvH reactivity amongst CBA fetuses and perinates. Throughout the entire period of observation, some degree of suppression of erythroid cell growth in irradiated Balb/c hosts was noted in lymphocytes from either the liver or the spleen. However, there was great variability in the magnitude of suppression as a function of age. There was a general elevation of the suppressive effect when a higher dose of allogeneic cells was used. Thus it was concluded that by day 7 post partum, GvH reactivity had not yet fully developed in CBA mice. This was not different from the low activity obtained during a comparable period of development in spleen colony recipients.

D. Discussion

The aim of this study was to assess the immunocompetence of developing lymphoid cells per se without the influence of other factors such as the maturity of the RES. The data presented show that when normal CBA fetuses and perinates were tested for GvH activity, both liver and spleen cell samples from donors between 15 to 26 days of age (post coitus) showed only a low degree of immunocompetence. In the *in vivo* cloning model, the recipients of cloned fetal liver cells also showed low GvH activity during a comparable period of development. The results suggest that the establishment of GvH reactivity requires 40-49 days of lymphoid differentiation starting from fetal liver stem cells. These data are in accordance with those of Dalmasso *et al.* (1963) who showed that GvH reactivity was not manifested in mice 2 weeks after birth. On the other hand, Fidler (1972) found that GvH reactivity is detectable in the newborn thymus and 3 days later, also in the spleen. Various workers have also demonstrated that cell-mediated transplantation immunity in mice appeared within a week after birth (Steinmuller, 1961; Argyris, 1965; Bortin, Rimm and Saltzstein, 1969). Recently, Hofman and Globerson (1973) have shown that yolk sac cells from 9-day old mouse embryos were able to elicit an *in vitro* GvH response. The discrepancy observed is most likely due to the differences in experimental systems, assays and mouse strains employed.

Strain variation in the pattern of immunological maturation has been reported by Playfair (1968) and Hechtel, Dishon and Braun, (1965).

GvH activity has been shown to be initiated by thymus-derived cells (Dalmasso et al. 1962; Cohen et al. 1963; Sosin, Hilgard & Martinez, 1966). Therefore the kinetics of development of GvH reactivity should parallel that of T-cells. Consistent with this view is the finding in this study that the onset of GvH reactivity (Fig.7) and that of humoral responsiveness to a T-dependent antigen, SRC (Fig.4b), fall within the same period of development. This suggests that the GvH effector T-cells may be identical with helper T-cells. Alternatively, they may be from two different T-cell lineages with the same rate of maturation. In the literature, there is evidence both for and against the first possibility. Gordon and Yu (1972) showed that mice which have developed cell-mediated immunity to SRC as measured by inhibition of spleen cell migration from capillary tubes in the presence of SRC, are deficient in helper T-cells required for the production of an antibody response to SRC. They concluded that the same population of T cells are endowed with both helper function and reactivity in cell-mediated immunity. This is contrary to the earlier findings of Segal, Cohen and Feldman (1972) who showed that hydrocortisone treatment in vivo abrogates helper T-cells in the induction of a humoral response to DNP but has no effect on T-cells needed in a GvH response. This controversy is

difficult to resolve in view of the heterogeneity within the T-cell population (Roitt et al. 1969; Meuwissen, Stuttman & Good, 1969) and the different assay systems used. Although the data of Segal and co-workers (1972) suggest that helper T-cells are different from T-cells involved in a GvH response, they do not prove that the effector cell types originate from two cell lineages. Raff and Cantor (1971) have proposed that different immunologic functions may be performed by T-cells going through various stages of maturation. Studies by Cantor (1972b) and Cantor and Asofsky (1970,1972) have demonstrated a synergy between two T-cell subpopulations in a cell-mediated GvH response. A hypothesis was put forward (Cantor, 1972a) that there are two classes of T-cells: a T1 cell which is capable of binding antigens, is non-recirculating and is found mainly in thymus and spleen; and a T2 cell which is recirculating and present in circulation and lymph nodes. It is postulated that the transition from T1 to T2 is driven by antigen, and that T2 cells act as amplifiers whereas T1 cells become the actual effectors in the GvH response (Cantor, 1972b). The data of Spear and Edelman (1974) favour the idea that the paucity of SRC response in vivo in perinatal Swiss L mice is due to the lack of a more mature population of T-cells, probably corresponding to the T2 cells of Cantor. Similar reasons may account for the results of the present study pertaining to the onset of the SRC response and of GvH reactivity.

Finally there is the possibility that the development of T-dependent immunological functions such as GvH reactivity and SRC responsiveness from cloned fetal liver stem cells in the present experimental model is limited by the rate of recovery of the thymus epithelium from the effect of irradiation. This would account for the similarity in the kinetics of the development of T-cell dependent immune responses. The use of thymus graft implantation may clarify this point.

CHAPTER VI

ONTOGENIC STUDIES ON IMMUNOLOGICAL TOLERANCE TO TRANSPLANTATION ANTIGENS

A. Introduction

A unique feature of the immune system is its ability to distinquish between self and non-self antigenic entities and to refrain from reacting against self tissue components. This tolerance towards 'self' is not due to the non-immunogenic nature of tissue antigens as they have been known to be potent immunogens when administered into other individuals of different antigenic compositions.

Immunological tolerance may also occur with respect to non-self antigens both naturally, as in red cell chimerism between dizygotic cattle twins (Owen, 1945) or under experimental conditions, as exemplified by chick parabionts (Hasek & Hraba, 1955) and tetraparental mice (Wegmann, Hellstrom & Hellstrom, 1971). Therefore, the same mechanism may be operative in both tolerance to self and non-self antigens.

Experimental tolerance is most readily induced during early ontogenesis at which time the embryo is still immunologically immature. Billingham, Brent and Medawar (1953) have shown that injection of adult allogeneic cells into fetuses <u>in utero</u> at 15 to 16 days of gestation led to almost 100% successful tolerance induction as tested by

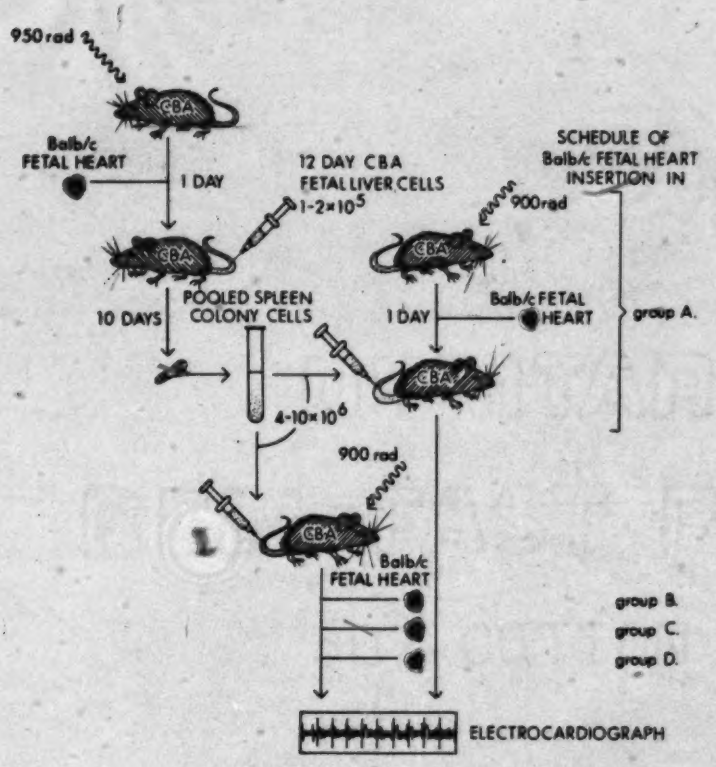

Figure 9 Experimental protocol for tolerance induction in recipients of cloned cells using cardiac allografts. Groups A to D differ in the time interval between the injection of cloned cells and heart allograft insertion.

acceptance of skin grafts of donor genotype when inserted 60 days after birth. Later experiments by Brent and Gowland (1961) showed that there was a decrease with age in the incidence of experimentally induced tolerance in newborn and perinatal mice.

The fact that self antigens are present from the first instance of lymphogenesis may be of importance in the establishment of self tolerance. Under experimental conditions, it is technically difficult to introduce an extrinsic antigen into the mouse fetus. The irradiated recipients of syngeneic stem cells have offered an experimental model whereby tolerance to an allograft may be studied during the process of immune reconstitution.

The following investigation has been carried out using two different experimental systems. Firstly, tolerance induction was attempted using the heart allograft model first introduced by Jirsch, Kraft and Diener (1974a). In this system, a fetal heart from an allogeneic donor is grafted onto the ear of an appropriate recipient. Provided this recipient is also irradiated and injected with syngeneic stem cells, induction of tolerance to the heart allograft may ensue during the process of immunogenesis. Fig.9 illustrates the experimental protocol in detail.

To further study the kinetics of tolerance during ontogenesis of the immune system, another experimental model has been used. When hemopoietic stem cells of a parent (CBA) strain are injected into irradiated CBF1 hybrids, the

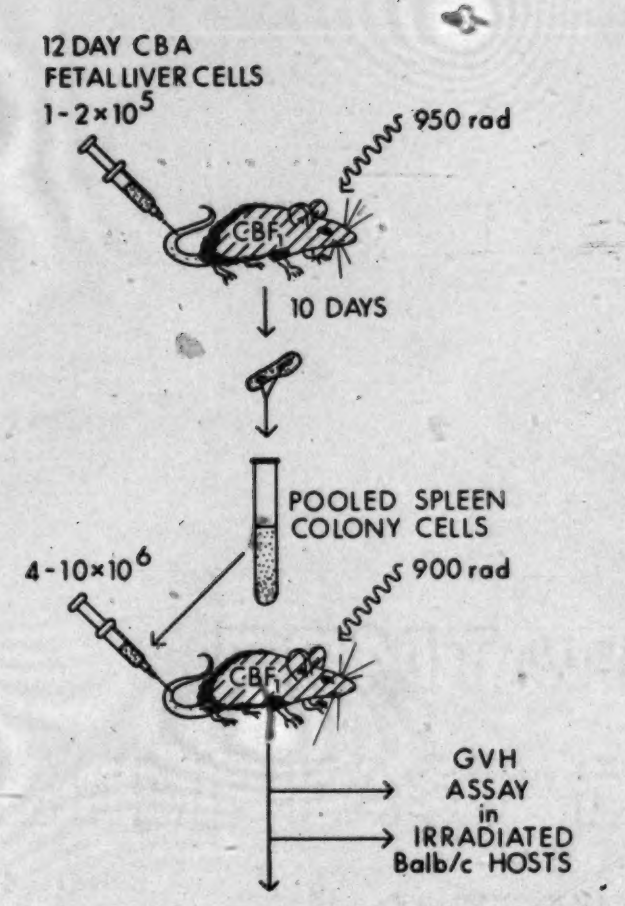

Figure 10 Experimental protocol for tolerance induction in radiation chimeras.
Parental CBA stem cells were injected into lethally irradiated (CBA x Balb/c)F1 hybrids (CBF1) in which they developed in the presence of Balb/c alloantigens. The state of tolerance in these chimeric mice was subsequently assessed by a GvH assay.

grafted cells are incapable of reacting against the antigens inherited from the other parental strain (Balb/c). These cells will, instead, proliferate and differentiate to reconstitute the hematolymphoid compartments of the recipients. Later on, host cell proliferation takes place and a state of chimerism results. Thus the CBA lymphocytes which mature in the CBF1 recipient must have become tolerant to Balb/c antigens present on host cells. This radiation chimera model was adopted in this study to induce tolerance to parental alloantigens. The optimal time of antigenic exposure during lymphoid development for tolerance induction was also investigated. The experimental protocol is summarized in Fig. 10.

B. Methods

1. GvH Assay and Evaluation of Results

Details of the GvH assay of Blomgren and Andersson (1972) have already been described in Chapter V. In addition to Balb/c, C57Bl/6J mice were also used as hosts, and since they were more radiation resistant, the radiation dose was increased to 850 rad. The number of CBA spleen cells necessary to produce effective suppression of C57Bl/6J erythropoiesis was 5×10^6 compared to 2×10^6 in Balb/c hosts.

2. Cytotoxicity Assay

The anti-Balb/c antiserum used has been kindly supplied

by Dr N.Kraft. It was raised by immunizing adult CBA mice with six intraperitoneal injections of 20 x 10^6 Balb/c spleen cells every other week. The mice were bled 10 days after the last injection. This antiserum was found to cause >90% Balb/c spleen cell death at a dilution of 1 : 32.

The cytotoxic activity of the anti-Balb/c serum on spleen cells from CBF1 recipients of CBA cells in the presence of guinea pig complement was assayed using the method of Terasaki and McClelland (1964). Mouse spleen cells were prepared as described, spun washed once in Leibowitz medium and resuspended in McCoy's 5a medium (GIBCO, Grand Island, N.Y.). The cells were then passed through a small column (approximately 8 x 30 mm) of fine glass beads pretreated with a 1% solution of gum arabic in deionized water (all from Fisher Scientific, Toronto) to remove polymorphonucleated cells. The latter take up eosin non-discriminantly and would distort the actual assessment of dead cells. The lymphocytes were eluted from the column with McCoy's medium and adjusted to a final concentration of 2 x 10^6 cells per ml.

One μl of serum at each dilution (five two-fold serial dilutions starting from neat serum) was added to each well of microtest plates (Terasaki micro-test plate #3034, Fisher Laboratories, Don Mills, Ontario) together with 1 μl of test cell suspension. The plates were incubated at room temperature for 30 min after which 5 μl of guinea pig complement (absorbed for an hour against an equal volume of

packed normal CBF1 erythrocytes) were added to each well. After a further 60 min incubation at room temperature, 3.3 µl of eosin (5% solution in distilled water) were added. Five minutes later, the cells were fixed with 8 µl of formaldehyde. Percent cell death was determined on the basis of eosin incorporation in triplicates for each serum dilution using an inverted phase contrast microscope. Control sera included a rabbit anti-Balb/c serum (positive control) and a normal CBF1 serum (negative control).

3. Cardiac Allografting and Subsequent Evaluation

Balb/c fetal hearts were obtained from embryos at 16-18 days of gestation. Cardiac allografting was performed by a modification of the technique of Fulmer et al. (1963) as described by Jirsch (1973). The allografts were inserted into the anterior aspects of one ear of the recipients according to the schedule below:

a) within 6 hr after irradiation (950 rad) of CBA primary recipients prior to fetal liver stem cell injection, and/or within 6 hr after irradiation (900 rad) of CBA secondary recipients prior to injection of spleen colony cells; and

b) at 1 to 20 days after spleen colony cells were injected into irradiated CBA recipients (Fig.9).

Allograft function following transplantation was

evaluated primarily by electrocardiography with visual confirmation of graft pulsation through the thin overlying skin of the external ear. Presence of allograft cardiac function was indicated by rhythmical electrical activity. Electrocardiographs (ECG) of allografts were recorded at weekly intervals starting any time between 3 to 7 days after grafting. The number of grafts showing cardiac activity at this time was used as the basis for calculating the subsequent percent graft survival.

In Jirsch's study (1973), it was found that mice which had successfully maintained an allograft for 12 weeks after irradiation and reconstitution would retain the graft indefinitely. Therefore they were considered tolerant to the graft. In the present study, a tolerant animal was also defined by the same criterion.

C. Results

1. Tolerance Induction in the Heart Allograft Model

Jirsch, Kraft and Diener (1974) have used a bone marrow fraction enriched for stem cells to reconstitute irradiated and heart-allografted mice. However, the cell separation technique used in their experiments could not completely remove bone marrow immunocompetent cells (Lafleur, Miller & Phillips, 1972) effective in allograft rejection which presumably interfered with tolerance induction. This prompted a study to test if the population of stem cells derived from hemopoietic spleen colonies would be more

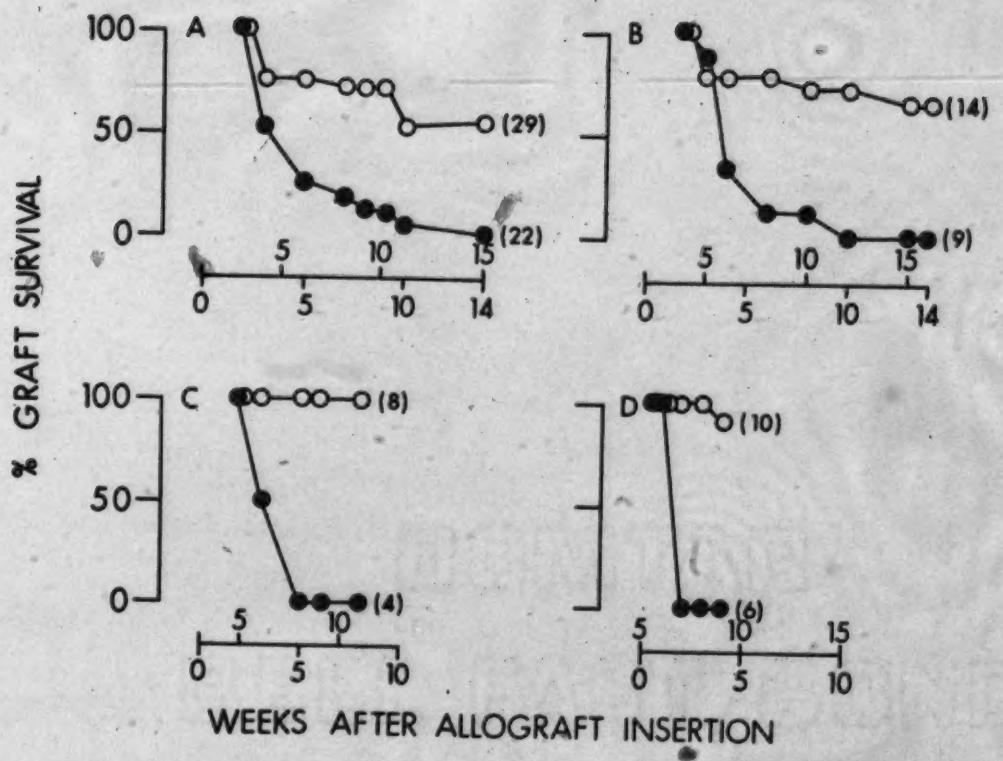

Figure 11 Survival of Balb/c heart grafts in irradiated CBA mice repopulated with cloned syngeneic fetal liver stem cells. The survival rate of these repopulated and allografted mice was also shown. The number of animals or established heart grafts per group at the beginning of the experiment is within parentheses. The upper scale of the abscissa represents the time in weeks after repopulation of the graft recipients with cloned cells. Groups A to D differ in the time interval between fetal liver stem cell repopulation and allograft implantation:

A -1 to 10 days
B 11 to 17 days
C 18 to 25 days
D 45 days

Surviving heart grafts (●-●)
Surviving graft recipients (o-o)

susceptible to tolerance induction since it has been shown that unlike stem cells, immunocompetent cells do not form spleen colonies (Nekori & Feldman, 1965). If this is indeed the case, there should have been improved allograft survival in recipients of cloned fetal liver stem cells. Furthermore, in this study, the correlation between the maturity of lymphoid cells and their susceptibility to tolerance induction was investigated. For this purpose, fetal liver repopulated hosts were given single cardiac allografts at various times after stem cell injection according to the experimental protocol shown in Fig.9. The state of tolerance in these mice was subsequently measured by the presence of functioning grafts.

Lethally irradiated, allografted and fetal liver reconstituted CBA mice were divided into four groups according to the time span between initial (in certain cases, the only) allograft insertion and fetal liver stem cell administration. The results are summarized in Fig.11.

At the end of 10 weeks following allograft insertion, only 1 out of 22 allografted mice in group A maintained a functioning Balb/c fetal heart. By week 14, cardiac function of this allograft was no longer detectable. Therefore no improvement of long term graft survival could be achieved by shortening the time span between allograft insertion and stem cell injection into irradiated recipients. Furthermore, there was a gradually intensifying tempo of rejection as the period between allograft insertion

and fetal liver cell injection lengthened. This effect was not due to differences in lymphoid maturity of the recipients at the time of rejection. For example, 100% (6/6) graft rejection was observed in group D mice 7 weeks after fetal liver repopulation. In contrast, at this same stage of lymphoid maturity, about 20% (4/22) of the grafts in group A were still functioning. It was therefore concluded that short term prolongation of graft survival was due to differences in the lymphoid maturity of the recipients at the time of allograft insertion.

Furthermore, on gross examination, the tempo of graft rejection in group D resembled that described for normal adult mice (Jirsch, Kraft & Diener, 1974b). This was interpreted as indicating that cell-mediated transplantation immunity was most likely established around 45 days of lymphoid development.

As a result of being frequently subjected to anaesthetization for electorcardiography, deaths occurred within the experimental groups amongst mice both with or without established allografts. Thus the observed percentages of functioning allografts are somewhat lower than the actual values. The contribution of the host's death to the outcome of survival time within a group was difficult to assess. To illustrate this point, the survival rate of host mice has been plotted alongside with the graft survival rate (Fig.11).

Previous studies by Jirsch, Kraft and Diener (1974b)

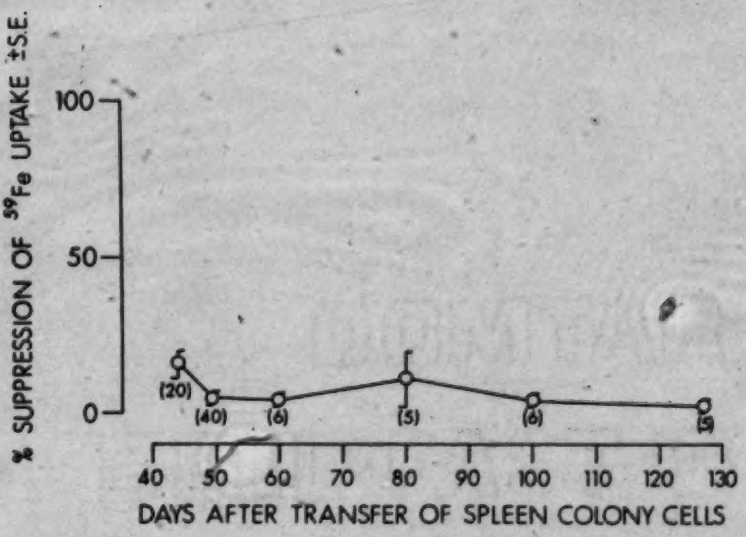

Figure 12 Development of anti-Balb/c activity in spleens of CBF1 recipients of cloned CBA cells. Anti-Balb/c activity was measured by the GvH assay as described in the legend of Figure 6. The number of Balb/c test mice per group is within parentheses.

have shown that in lethally irradiated, bone marrow repopulated and heart allografted mice, no graft was maintained for longer than 10 weeks. It is therefore concluded that susceptibility to tolerance induction is similar for both fetal liver and bone marrow hemopoietic stem cells.

2. Tolerance Induction in the Cell Chimera Model

It was reasoned that parental lymphoid cells might more readily become tolerant to alloantigens of the opposite parent if allowed to develop in lethally irradiated F1 hybrids. Thus stem cells from CBA fetal livers (12 days of gestation) were allowed to proliferate and differentiate in the continual presence of Balb/c histocompatibility antigens by placing them in CBF1 hybrids using the protocol outlined in Fig.10. Again anti-Balb/c activity was measured by a GvH assay as described in Chapter V. The results are shown in Fig.12.

When differentiation of CBA fetal liver stem cells was allowed to take place in lethally irradiated CBF1 mice, none of the 17 treated mice tested between 44 to 127 days after the injection of cloned cells exhibited anti-Balb/c activity. In contrast to this result, CBA fetal liver stem cells after a total of 60 days of differentiation and maturation in irradiated syngeneic hosts gave rise to cells in the spleen which were capable of eliciting a GvH response in irradiated Balb/c hosts (Fig.7).

TABLE 6 Cytotoxicity of CBA anti-Balb/c serum for spleen cells from CBF1 recipients of cloned CBA cells[1]

Serum (undiluted)	Cytotoxicity value of cells from (Each figure represents one well)			
	CBF1	CBA	Balb/c	test[2]
Normal Balb/c	111	111	111	111
Normal CBA	111	111	111	111
Rabbit anti-Balb/c	888	888	888	888
Balb/c anti-CBA	888	888	111	888
CBA anti-Balb/c	888	111	888	111

Cytotoxicity value: 1 - cytotoxicity <10% cells
4 - cytotoxicity >10%<25% cells
6 - cytotoxicity >25%<80% cells
8 - cytotoxicity >80% cells

1 Each CBF1 was irradiated (900 rad) and given 4 - 10 x 10^6 cells from pooled 10-day old spleen colonies derived from CBA fetal liver stem cells.

2 One CBF1 recipient of cloned CBA cells was killed 100 days after repopulation and its spleen cells were used in this test.

TABLE 7 Cytotoxicity of CBA anti-Balb/c serum for spleen cells from CBF1 recipients of cloned CBA cells[1]

Serum (dilution)	Cytotoxicity value[2] in test mice[3] (Each figure represents one well)			
	1	2	3	4
normal CBF1 (0)	111	111	111	111
rabbit anti-Balb/c (0)	880[4]	888	880	880
CBA anti-Balb/c (0)	111	111	111	111
(1/2)	411	111	111	111
(1/4)	111	114	111	111
(1/8)	111	111	111	111
(1/16)	111	111	111	111
(1/32)	111	111	111	111

1 See legend 1 in Table 6.

2 See legend in Table 6.

3 Four CBF1 recipients of cloned CBA cells were killed 127 days after repopulation and the spleen cells from individual animals were assayed for sensitivity to anti-Balb/c serum in the presence guinea pig complement.

4 Too few cells

3. Origin of Cells in CBF1 Recipients of Cloned CBA Cells

An anti-Balb/c alloantiserum raised in CBA mice was used to test the origin of the lymphoid cells in the spleens of CBF1 mice repopulated with CBA cells. Five spleens were tested 110 and 137 days after stem cell transfer. Three of them gave a cytotoxicity value of 1 (Tables 6 and 7), an equivalent of 0 to 10% cell death, following incubation with anti-Balb/c serum at dilutions of 0 to 1/32 in the presence of guinea pig complement. In two mice, a cytotoxicity value of 4 was observed in one of the triplicates at serum dilutions of 1/2 and 1/8 respectively. A cytotoxicity value of 1 was observed when non-specific target cells or antisera were used. Therefore it was concluded that the donor CBA lymphoid cells persisted in the CBF1 radiation chimeras and that at the most, the hosts contributed 10% of the total spleen lymphoid cells up to 137 days of development.

4. Specificity of Tolerance in CBF1 Radiation Chimeras

Two different mechanisms could be responsible for the lack of anti-Balb/c activity in CBF1 radiation chimeras. Firstly, it could represent the establishment of a true state of tolerance specific for Balb/c histocompatibility antigens. Secondly, it could result from a state of general lack of transplantation immunity. To distinguish between these two possibilities, specificity of tolerance was tested using a 'third party' strain, C57Bl/6J mice as hosts in the GvH assay. Results are shown in Table 8.

TABLE 8 Anti-Balb/c and anti-C57Bl/6J activity[1] in CBF1 recipients of cloned CBA cells[2]

Days after transfer of cloned cells	Spleen cell dose ($\times 10^6$)	Mean % suppression ± S.E. (No. of Mice)	
		Balb/c	C57Bl/6J
127	2	2.0 ± 2.0 (5)	30.3 ± 13.2 (6)
	10	0 (4)	92.7 (1)[3]
194	3	N.T.	9.3 ± 5.9 (5)
	6	N.T.	73.6 ± 7.4 (5)[4]
	7.5	N.T.	93.2 ± 1.1 (4)[4]

1 This was assayed by a GvH assay as described in legend 1, Table 5.

2 See legend 1, Table 6.

3 There were originally 6 animals in this group but 5 of them died from severe GvH disease by the time of ^{59}Fe injection.

4. Significant suppression, $P<0.001$

N.T. not tested

Normally, 2 x 10^6 CBA spleen lymphocytes are sufficient to produce a significant ($p<0.05$) suppression of Balb/c erythropoiesis in irradiated hosts. However, as many as 10 x 10^6 spleen cells recovered from a CBF1 radiation chimeric mouse failed to suppress any Balb/c erythropoiesis although significant suppression was produced in irradiated C57Bl mice. This was confirmed in another experiment. Therefore, it was concluded that transplantation immunity remained intact in the CBA-CBF1 radiation chimeras, and that specific tolerance had developed to Balb/c alloantigens.

5. Adoptive Transfer of Tolerance to Balb/c Heart Allografts

It has been shown that cells from the CBF1 radiation chimeras were non-reactive against Balb/c alloantigens. To test if specific tolerance could be transferred by these cells, 6.1 x 10^6 lymph node cells from CBF1 mice reconstituted with CBA cells were injected into irradiated (950 rad) CBA recipients each of which had received 2 x 10^5 syngeneic bone marrow cells and a Balb/c fetal heart graft. The allografts in these animals survived up to 15 weeks, thereafter allograft activity could not be followed due to the death of the animals. Since in the allograft model a graft survival time of 12 weeks has been taken to be the cut off point to delineate tolerance from immunity, these animals were regarded as tolerant to the allograft. Although 8.2 x 10^6 normal CBF1 lymph node cells were found

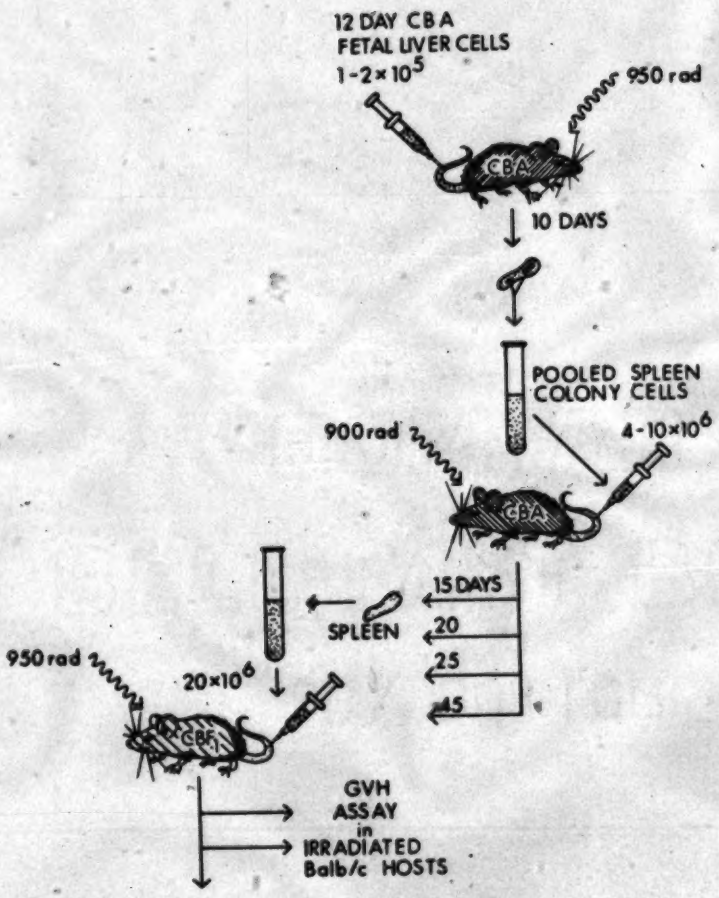

Figure 13 Experimental protocol to study susceptibility to tolerance induction during lymphoid development in parent-F1 radiation chimeras towards alloantigens of the opposite parent.

to have a similar effect, the degree of sensitivity to anti-Balb/c antiserum and guinea pig complement suggests a maximal contamination of only 10% CBF1 cells in the radiation chimeras. This is compatible with the notion that donor cells in the reconstituted CBF1 mice are responsible for the adoptive transfer of tolerance in irradiated CBA animals.

6. Susceptibility to Tolerance Induction during Lymphoid Development

The relationship between immunological maturity of lymphoid cells and their susceptibility to tolerance induction was examined. CBA fetal liver cells which had undergone initial differentiation in irradiated syngeneic hosts for 25, 30, 35 or 55 days were exposed to Balb/c antigens upon transfer into irradiated CBF1 hosts (Fig.13). The state of tolerance to Balb/c alloantigens was measured by subsequent evaluation of anti-Balb/c activity in the spleens between 56 to 147 days after repopulation of the CBF1 hosts. The results are shown in Fig.14. When the period of initial development in CBA hosts was 25, 30 or 35 days, spleen cells from the final CBF1 hosts showed significantly ($p<0.001$) lower anti-Balb/c activity in comparison with that observed when CBA mice were used as the final hosts. These results suggest that even after 35 days of lymphoid development, prolonged exposure to alloantigens can still lead to unresponsiveness.

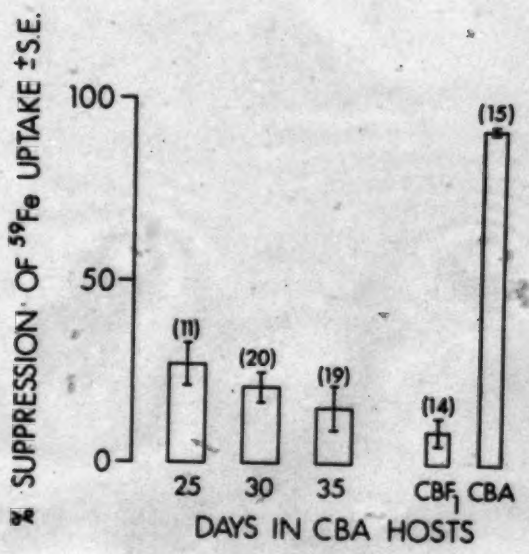

Figure 14 Anti-Balb/c activity of splenic lymphocytes recovered from irradiated CBF1 mice repopulated with CBA fetal liver derived lymphoid cells which had undergone the first 25, 30 or 35 days of differentiation in irradiated CBA hosts.
Anti-Balb/c activity was measured by the GvH assay as described in the legend of Figure 6. Each CBF1 recipient was reconstituted with 20×10^6 spleen cells from CBA mice repopulated previously with cloned CBA stem cells. The number of Balb/c test mice per group is within parentheses. CBF1 and CBA control mice were irradiated and repopulated with 20×10^6 spleen cells recovered respectively from irradiated CBF1 and CBA mice reconstituted with cloned CBA stem cells 30 days before killing.

When lymphoid cells which had undergone 55 days of development in irradiated CBA hosts were transferred into irradiated CBF1 recipients, the latter (a total of 12 mice) all died within 18 days after cell injection, presumably as a result of a GvH response. This suggests that immunocompetence is established within 55 days of lymphoid development and is in agreement with results presented in the previous chapter. Furthermore, since unresponsiveness can still be induced by day 35 of lymphoid development, the results of this study are in support of the idea that a tolerance susceptible period precedes the onset of immunocompetence during immune ontogenesis.

D. Discussion

Immune development of fetal liver stem cells in irradiated syngeneic hosts bearing cardiac allografts did not result in tolerance towards the grafts whereas development in irradiated F1 hybrid mice induced tolerance against the alloantigens of the opposite parent. Various factors could contribute to this discrepancy. Firstly, the allograft represents a limited and localized source of antigen. Therefore unsuccessful tolerance induction in the allograft model may be explained on the basis of dose effect (Billingham & Brent, 1959; Brent & Gowland, 1961). In contrast, the CBF1 corporal milieu provides an environment with abundant, widely accessible Balb/c alloantigens, conducive to tolerance induction. Secondly, initial

proliferation and differentiation of hemopoietic stem cells is apparently restricted to the spleen and bone marrow rather than to the circulatory system (Micklem & Loutit, 1966). In the allograft model, this would render the encounter between antigens and the developing lymphoid cells difficult and differentiation of cloned CBA fetal liver stem cells towards immunocompetence might not have occurred in the presence of Balb/c antigens. Thirdly, Balb/c histocompatibility antigens may have been shed by cells from the heart graft and then brought into contact with the developing CBA lymphoid cells via circulation. This is supported by the fact that in man, soluble histocompatibility antigens have been reported to be released from cells into the serum (Charlton & Zmijewski, 1970). Therefore CBA lymphocytes might have become tolerant to Balb/c histocompatibility but not to heart tissue antigens. Graft rejection could then be explained as a result of reactivity against heart specific antigens. It seems unlikely that heart allograft survival is a more sensitive method for evaluating the state of tolerance than is the GvH assay since preliminary data show that lymph node cells from the chimeric CBF1 mice (found to be tolerant by the GvH assay) were also able to adoptively transfer tolerance to Balb/c heart grafts in irradiated CBA mice repopulated with syngeneic bone marrow.

The allograft rejection frequency observed in the present study paralleled that reported by Jirsch and co-

workers (1974b) in a similar study using purified stem cells from adult bone marrow to repopulate irradiated mice. These authors reasoned that there were still some immunocompetent cells in the stem cell enriched fraction of the bone marrow after separation by velocity sedimentation which caused allograft rejection. The possibility of cells competent in allograft rejection amongst cloned fetal liver cells cannot be excluded. However, evidence to date is compatible with the view that immunocompetent cells do not form spleen colonies (Mekori & Feldman, 1965).

The data presented show that allografts survived better when they were implanted earlier during lymphoid development in irradiated recipients of cloned cells. Consistent with this are the data obtained using the cell chimera model which also suggest that there is a tolerance susceptible period during the development of lymphoid cells prior to the onset of immunocompetence.

The ease of tolerance induction in newborn animals has been attributed to their low phagocytic activity (Nossal & Mitchell, 1966). In this study, a similar situation may occur during immune development in the irradiated recipients of cloned fetal liver cells. Indirect evidence supporting the idea that irradiated mice are deficient in phagocytosis has been provided by Gorczynski, Miller and Phillips (1971). They showed that functional adherent cells, which are essential in a SRC response (Shortman et al, 1970), are absent from the spleens of irradiated mice 3 days after

irradiation. Adherent cells are shown to have phagocytic activities and are probably macrophages (Shortman et al. 1970). An equally likely explanation for the greater ease of tolerance induction during early ontogeny is that less mature lymphoid cells require a lower dose of antigens for tolerance induction. Studies by Nossal (1974) have shown that cells from fetal mice were tolerized by a dose of antigen 10-fold lower than that required to tolerize adult cells.

Lymph node cells from tolerant chimeric animals were able to adoptively transfer tolerance to Balb/c heart allografts when injected together with CBA bone marrow cells into irradiated CBA recipients. This is in agreement with the results of Jirsch, Kraft and Diener (1974b) using lymph node cells from mice tolerant to heart allografts. These data suggest that specific suppressor cells may be present in tolerant animals.

CHAPTER VII

DEVELOPMENT OF RESPONSIVENESS TO PHYTOMITOGENS. INFLUENCE OF THE THYMUS AND RELATIONSHIP TO IMMUNOLOGICAL COMPETENCE

A. Introduction

Lymphocytes can be induced by phytomitogens to undergo blast transformation *in vitro*. The gross morphological and biochemical characteristics of such a response resemble those of specific antigen induced immune reactions (Coulson & Chalmers, 1964; Chessin *et al*. 1966). Studies with cells from individuals with selective immunological impairment of thymic function have shown that responsiveness to phytohemagglutinin (PHA) is predominantly a property of T-cells (Oppenheim, 1968; Douglas, Kamin & Fudenberg, 1969; Rodey & Good, 1970). Using pokeweed mitogen (PWM), others have shown that activated cells develop ultrastructural characteristics of plasma cells *in vivo* - a pattern of response specific for B-cells (Douglas *et al*. 1967; Barker, Lutzner and Farner, 1969). Earlier studies on mouse lymphocyte responsiveness to mitogens *in vitro* have led to the conclusion that PHA is T-cell specific whereas PWM stimulates B-cells exclusively (Janossy & Greaves, 1971; Stockman *et al*. 1971). Later, Janossy and Greaves (1972) showed that PWM also stimulates T-cells. Furthermore, Greaves and Bauminger (1972) have demonstrated that although

soluble PHA stimulates T-cells only, insoluble PHA (bound to 'Sepharose' beads) can induce DNA synthesis in both T- and B-cells.

Although soluble mitogens have selectivity for different lymphocyte classes, their action is non-specific. In other words, a mitogen can stimulate a large proportion of lymphocytes regardless of their antibody specificities. Nevertheless, the initial event in specific (by antigens) or non-specific (by mitogens) lymphocyte activation involves the combination of the stimulating ligand with receptors on lymphocytes (Byrt & Ada, 1969; Greaves, Bauminger & Janossy, 1972).

Because of the specificity of phytomitogens with respect to lymphocyte classes, they may be used to study the ontogenic development of T- and B-cells. The present study compares the development of mitogen responsiveness with that of immunocompetence in irradiated, bone marrow repopulated mice. The role of thymus in the development of these characteristics was also examined. In addition, an attempt was made to investigate possible changes in physical characteristics of developing PHA and PWM responsive cells using cell separation by velocity sedimentation techniques.

B. Methods

1. Cell Separation

At the time of assay, groups of 4 to 5 mice were killed by chloroform inhalation and bled from the heart to reduce

the erythrocyte content of the spleen. Spleen cell suspensions were prepared in PBS containing 3% FCS. No more than 4 x 10^8 total cells were placed in the top layer of 45 ml of 3%FCS in PBS of a 900 ml gradient. Velocity sedimentation separation was performed using a 'staput' apparatus according to the method of Miller and Phillips (1969). The cells were allowed to sediment for 2 hr at 4°C. Fractions of 15 ml were collected and cells were counted using a Coulter Model B cell counter (Coulter Electronics, Inc., Hialech, Fla.). Sedimentation velocities were calculated using a computer program as described by Kraft and Shortman (1972). Where staput fractions contained few cells, adjacent fractions were combined to provide a minimum number of 1 x 10^6 cells per culture. The cell suspensions were spun down and the pellets resuspended in culture medium and placed in tissue culture.

2. Mitogens

PHA was obtained in powder form (MR10, Burroughs, Wellcome, England). Pokeweed mitogen powder was obtained from Grand Island Biological Corporation (Grand Island, N.Y.). Solutions of mitogens were prepared as instructed, stored at -20°C, and used within 2 weeks.

3. Tissue Culture

Spleen cells before and after separation were cultured in Eagle's minimal essential medium (MEM) supplemented with 10% heat inactivated FCS and 100 units/ml of penicillin G

and 100 µg/ml of streptomycin (all from GIBCO, Grand Island, N.Y.). The medium was gassed with CO2 to pH 7.2 before use. The number of cells per culture ranged from 1 to 4 x 10^6 suspended in 2.5 ml of medium. Cell suspensions were placed in sterile, disposable glass tubes (12 x 75 mm) fitted with loose tin-foil caps. Cultures were placed in a humidified incubator at 37°C and gassed with a mixture of 8% O2, and 10% CO2 in nitrogen. The maximum culture period was 96 hr. Triplicate cultures were set up in (a) medium alone; (b) medium with 10 µl/ml of PHA; and (c) medium with 8 µl/ml of PWM. One µCi/ml tritiated thymidine (^{3}H-TdR) (New England Nuclear, Boston, Mass.) was added for the terminal 6 hr of culture. At the termination of the cultures, they were placed in an ice bath and the cells harvested for determination of the amount of thymidine incorporated into DNA.

4. Estimation of Thymidine Incorportion into DNA

Cells were harvested by centrifugation at 330g for 10 min at 4°C, and the supernatant discarded. DNA was extracted from the cell pellet by cold acid precipitation. 5 ml of cold 5% trichloroacetic acid (TCA) were added to each tube and the contents were mixed in a Vortex mixer. The suspensions were allowed to stand at 4°C for 20 min. The pellet was recovered by centrifugation at 660g for 10 min at 4°C and dissolved in 0.8 ml tetraethylammonium hydroxide, (10% in water, Eastman Kodak Co., Rochester,

N.Y.) and transferred into scintillation counting vials by mixing with 10 ml of scintillation mixture (Bach & Voynow, 1966) consisting of:

xylene	468 ml
dioxane	468 ml
100% ethanol	280 ml
naphthalene	98.4 g
2,5-diphenyloxazole	6.13 g
α-naphthylphenyloxazole	0.0613 g.

Tritium counts were obtained using a Packard 2420 Tricarb Liquid Scintillation Spectrometer (Packard Instrument Co., Downers Grove, Il.).

5. Determination of Humoral Immune Responsiveness

The techniques have been described in detail in Chapter III. At different times following repopulation, groups of 3 or 4 mice were immunized with POL and SRC. Each test included a normal adult mouse as control. The number of PFC to either antigen in the spleen was determined 4 days later.

6. Expression of Results

a. Humoral immune response

The number of PFC per spleen was calculated and the mean of each group was taken. This number was expressed as the percentage of normal control values obtained in adult immunized CBA mice.

b. Response to mitogens

The mean 72 hr response (of triplicate cultures) to mitogens of unfractionated spleen cells was quantitated as mean cpm per spleen above background (unstimulated culture). This number was expressed as a percentage of the mean response observed in a normal spleen. The latter had been determined from 25 experiments to be:

PHA: 1,736,223 ± 21410 cpm/spleen

PWM: 595,707 ± 21412 cpm/spleen.

For fractionated spleen cells, the response in each pool of fractions was expressed as mean absolute cpm above background (unstimulated) per velocity increment per spleen. To illustrate how this value can be derived, let

a = mean cpm per stimulated culture,

b = mean cpm per unstimulated culture,

n = number of cells per culture,

N = number of cells from the pooled fractions,

x = number of spleens used for fractionation,

v = velocity difference in mm/hr between the first and the last fractions of this pool.

Then the mean response per culture = a - b,

the mean response per cell = (a - b)/n, and

the mean response of the pool = N(a - b)/n.

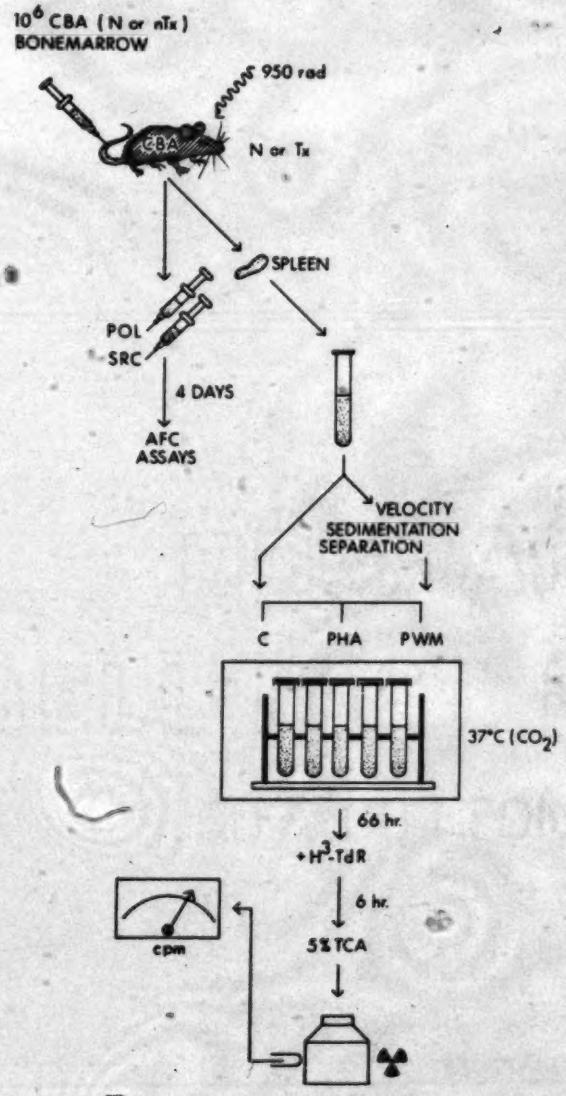

Figure 15 Experimental protocol to study the development of humoral immune responsiveness and mitogen responsiveness in whole or fractionated spleen cells from irradiated, syngeneic bone marrow repopulated CBA mice.

Therefore the mean response of the pool per velocity increment per spleen is

$$= \frac{N(a - b)/n}{vx} \quad cpm$$

To determine the sedimentation velocity distribution profile, the mean absolute response per velocity increment per spleen was determined for each pool of fractions and plotted against the average sedimentation velocity. The resulting graphs were normalized to the same peak height taken as 100%, regardless of the absolute numbers.

C. Results

In this study, the development of mitogen and humoral immune responsiveness in irradiated, bone marrow repopulated mice was followed according to the experimental protocol outlined in Fig.15. The role of the thymus on the development of these activities was investigated from two aspects. Firstly, the thymic influence on the precursors of cells participating in humoral and mitogenic responses was studied using bone marrow cells from neonatally thymectomized (nTx) donors. Secondly, the thymic influence on the development of mitogen and humoral immune responsive cells was examined using mice thymectomized at 15 days of age (Tx) as recipients. Non-thymectomized adult mice (N) were used as bone marrow donors or recipients for comparison. There were 4 marrow-recipient combinations in

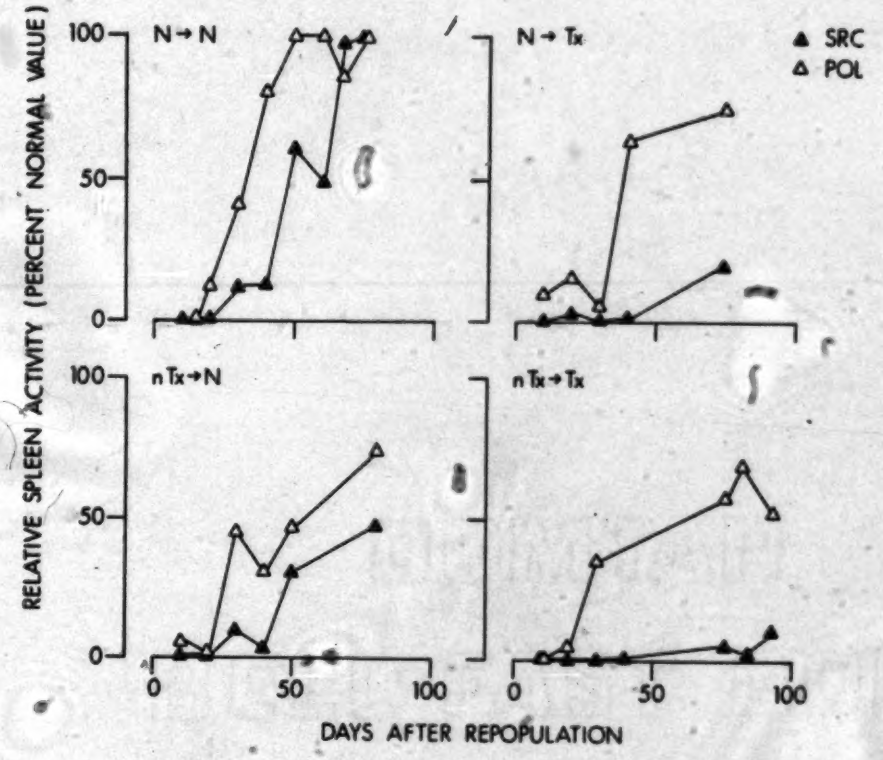

Figure 16 Development of humoral immune responsiveness in irradiated, syngeneic bone marrow repopulated CBA mice.
Each point represents the mean of 2 to 4 mice.
Each assay included one normal control mouse.
Anti-POL AFC Δ-Δ
Anti-SRC AFC ▲-▲

- N→N normal bone marrow into non-thymectomized recipients
- nTx→N bone marrow from neonatally thymectomized donors into non-thymectomized recipients
- N→Tx normal bone marrow into thymectomized (15 days of age) recipients
- nTx→Tx bone marrow from neonatally thymectomized donors into thymectomized recipients

total: N->N; nTx->N; N->Tx; and nTx->Tx. The results from these 4 groups are considered en bloc for each criterion of development studied.

1. Development of Humoral Immune Responsiveness to POL and SRC

The development of humoral immune responsiveness to POL and SRC was followed in irradiated, bone marrow repopulated CBA mice. At various times following repopulation, groups of 3 or 4 mice were immunized with POL and SRC and the number of spleen PFC to either antigen was assayed four days later. The results are shown in Fig.16.

In N->N mice, anti-POL responsiveness started to increase on day 20 after bone marrow repopulation reaching normal levels by day 50. The other three groups of repopulated mice: nTx->N, N->Tx, and nTx->Tx, showed a similar pattern in the recovery of anti-POL responsiveness although only 75% of the normal control value was reached by day 75. These data suggest that thymectomy of either the bone marrow donor or the recipient has an adverse effect on the rate of restoration of humoral responsiveness to POL, a T-cell independent antigen.

Responsiveness to SRC remained low in all four groups of experimental animals for 40 days following bone marrow repopulation. Thereafter, it increased progressively in N->N and nTx->N mice reaching 100% and 50% of the normal level respectively by day 75. This suggests that neonatal

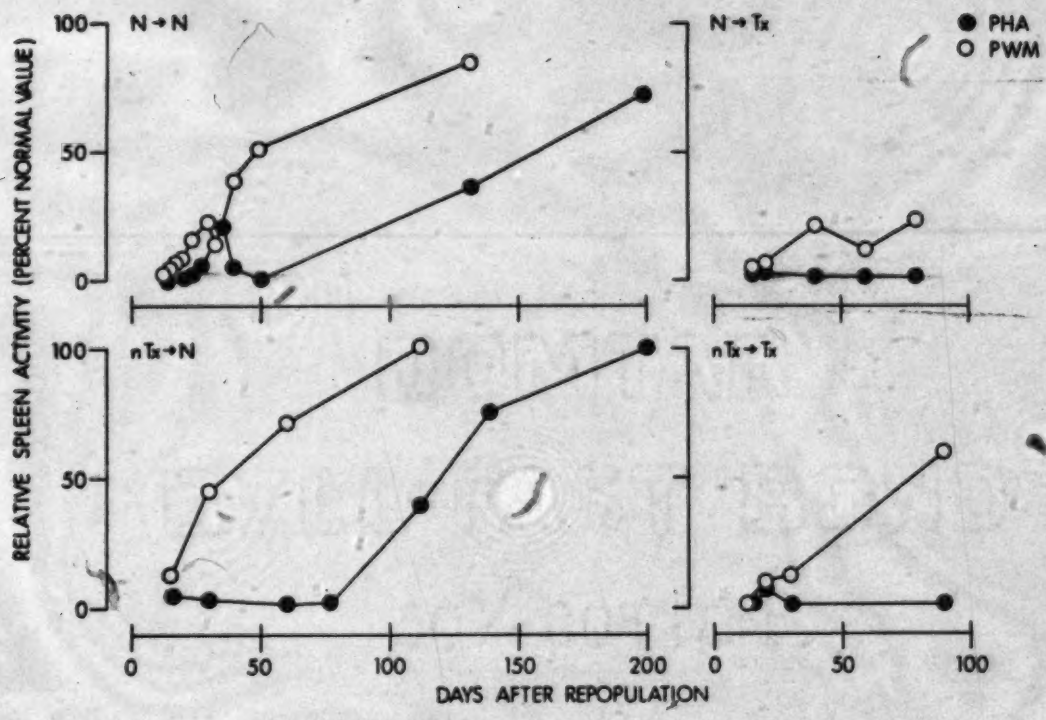

Figure 17 Development of responsiveness to PHA and PWM in irradiated, syngeneic bone marrow repopulated CBA mice.
Each point represents one experiment performed using cells from a pool of 4 or 5 spleens.
PHA responsiveness ●–●
PWM responsiveness o–o
N→N, N→nTx, nTx→N, nTx→Tx represent different bone marrow-recipient combinations as described in the legend of Figure 16.

thymectomy of the bone marrow donor can also influence the recovery of the anti-SRC response. In the remaining 2 groups of mice; N->Tx and nTx->Tx, the responsiveness to SRC was below 20% of the normal value up to day 90, as may be expected of a T-dependent antigen.

2. Development of Responsiveness to PHA and PWM

The responsiveness of irradiated, bone marrow repopulated mice to PHA and PWM was tested at various times following bone marrow repopulation using cells pooled from 4 or 5 spleens. The cells were cultured alone or in the presence of either PHA or PWM for 72 hr. Tritiated thymidine was added to all cultures 6 hr prior to the termination of culture and mitogen responsiveness was measured by assessing the incorporation of tritiated thymidine into DNA. The results are shown in Fig.17.

Responsiveness to PWM was first detectable in the spleens of all experimental animals by day 15 after bone marrow repopulation. Thereafter, the development of PWM responsiveness differed between N and Tx recipients. Both N->N and nTx->N mice showed a faster rate of recovery when compared with N->Tx and nTx->Tx mice. Thus by day 50, the level of PWM responsiveness in N->N mice was 50% of the normal value and that in nTx->N mice was 65%, whereas in N->Tx and nTx->Tx mice, the responsiveness to PWM by day 50 was 20 and 30% respectively of the normal control value. Normal levels of responsiveness to PWM was reached in nTx->N mice

by day 110. These results suggest that the development of PWM responsiveness was slower in thymectomized recipients.

Responsiveness to PHA, a T-cell specific mitogen, was negligible during the first 50 days after bone marrow repopulation. Thereafter it increased progressively in non-thymectomized recipients and by day 200, the level of PHA responsiveness reached the normal control value in nTx→N mice but was only 70% of this value in N→N animals. Apart from a low level of PHA responsiveness by day 20 in nTx→Tx mice, no responsiveness to PHA was detectable in N→Tx or nTx→Tx mice up to day 80 or 90 after repopulation. The response to PHA was not followed beyond this time point.

3. Sedimentation Velocity Distribution Profiles of PHA and PWM Responsive Spleen Cells at Different Stages of Development

The cells used in this study were from the same suspension pooled from 4 or 5 spleens of bone marrow repopulated mice prepared for use in experiments described in the preceeding section.

In order to have at least 10^6 cells per culture, cell fractions collected after separation were appropriately combined into several pools. Their response to PHA and PWM *in vitro* was assayed by the incorporation of tritiated thymidine into DNA. The results are shown in Figs. 18 to 21. Although the results for each donor-recipient combination at each time point are from one experiment, the

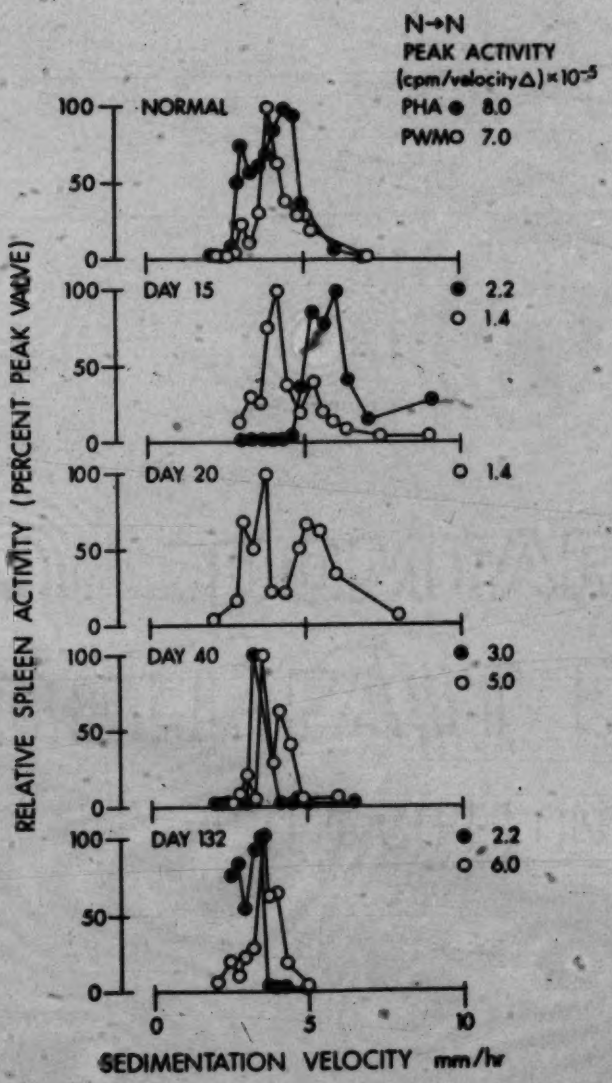

Figure 18 Sedimentation velocity distribution profiles of PHA and PWM responsive cells in irradiated, syngeneic bone marrow repopulated (N→N) CBA mice.
At different times after repopulation, one experiment was performed using a pooled cell suspension from 4 or 5 spleens.
PHA responsive cells ●–●
PWM responsive cells o–o
N→N bone marrow from normal donors into non-thymectomized recipients

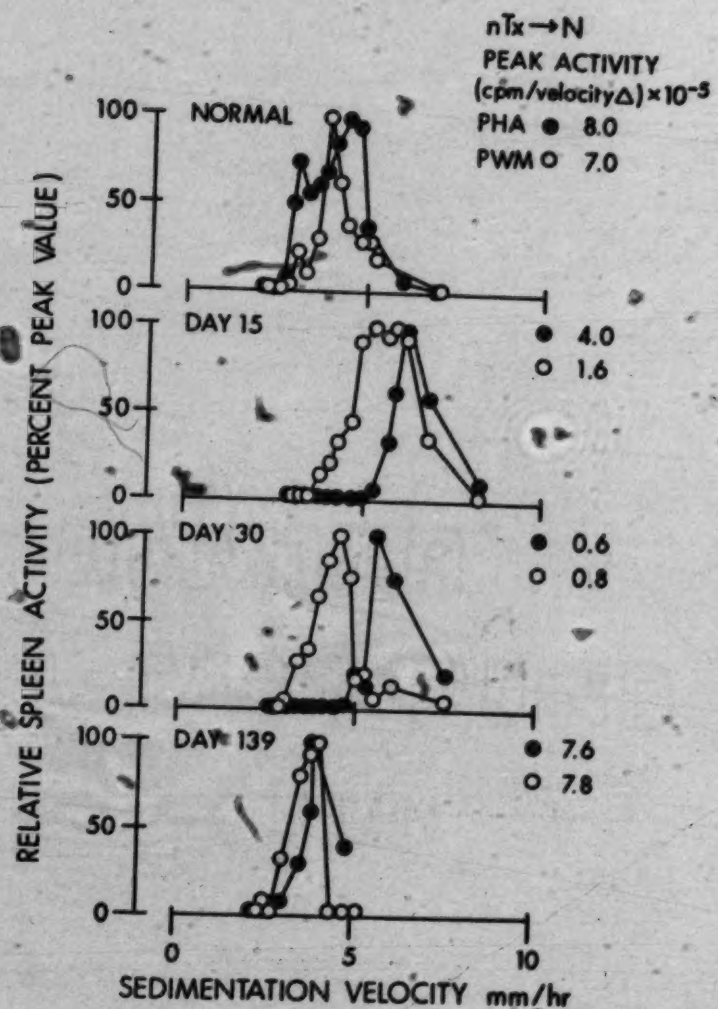

Figure 19 Sedimentation velocity distribution profiles of PHA and PWM responsive cells in irradiated, syngeneic bone marrow repopulated (nTx→N) CBA mice.
At different times after repopulation, one experiment was performed using a pooled cell suspension from 4 or 5 spleens.
PHA responsive cells ●–●
PWM responsive cells o–o
nTx→N bone marrow from neonatally thymectomized donors into non-thymectomized recipients

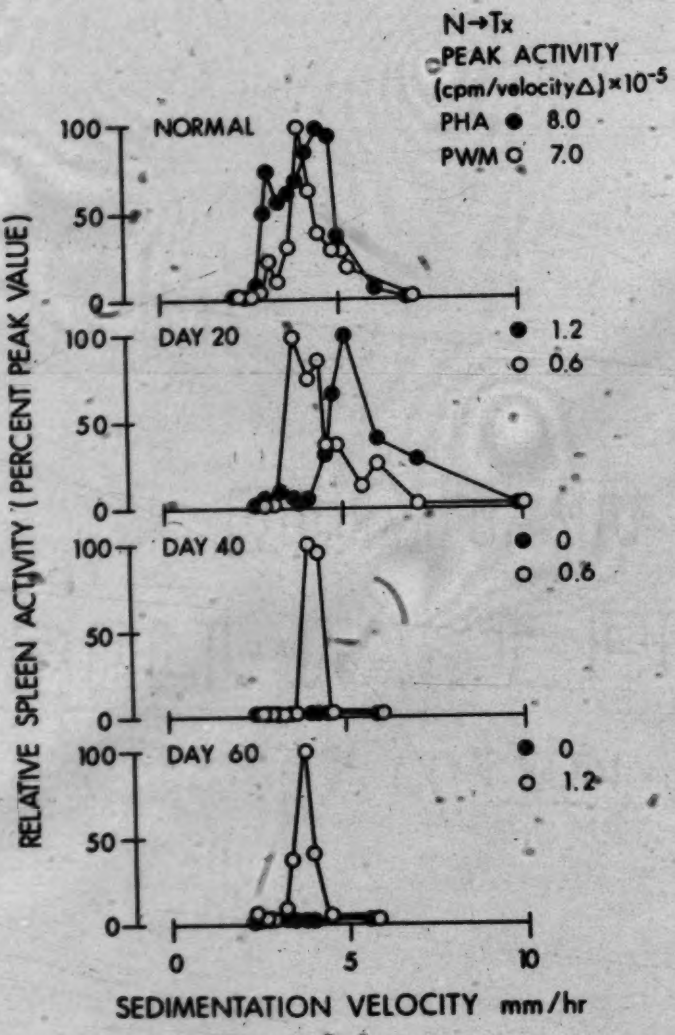

Figure 20 Sedimentation velocity distribution profiles of PHA and PWM responsive cells in irradiated, syngeneic bone marrow repopulated (N→Tx) CBA mice.
At different times after repopulation, one experiment was performed using a pooled cell suspension from 4 or 5 spleens.
PHA responsive cells ●-●
PWM responsive cells o-o
N→Tx normal bone marrow into thymectomized (at 15 days of age) recipients

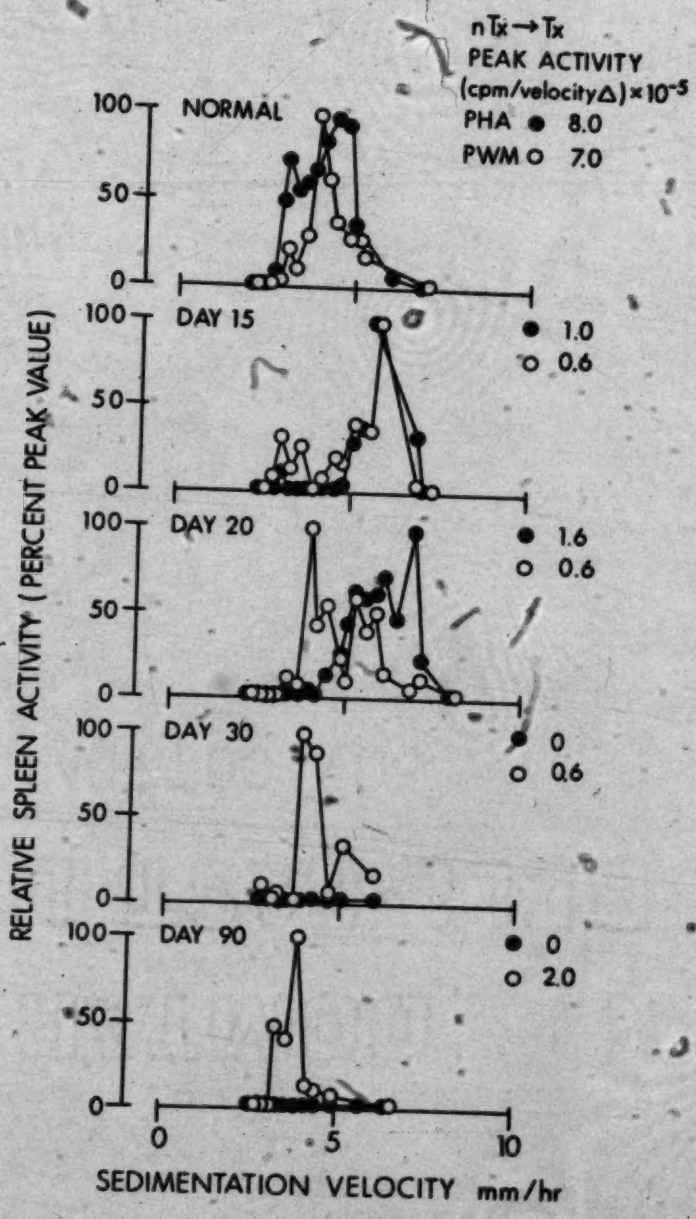

Figure 21- Sedimentation velocity distribution profiles of PHA and PWM responsive cells in irradiated, syngeneic bone marrow repopulated (nTx→Tx) CBA mice.
At different times after repopulation, one experiment was performed using a pooled cell suspension from 4 or 5 spleens.
PHA responsive cells ●–●
PWM responsive cells o–o
nTx→Tx bone marrow from neonatally thyemctomized donors into thymectomized (at 15 days of age) recipients

reproducibility of the major peaks of mitogen responsive cells in different experiments argues against their being artifacts.

In normal spleens, the sedimentation velocity distribution of cells responsive to PHA and PWM formed two partially overlapping series of peaks. The major peak of PHA responsive cells extended over a relatively wide range with a mean sedimentation velocity value of 4.4 mm/hr. The major peak of PWM responsive cells was a narrower band around a sedimentation velocity value of 3.9 mm/hr.

In irradiated, bone marrow repopulated animals, the sedimentation velocity distribution profiles of both PHA and PWM responsive cells changed as a function of time. The general pattern of change was a shifting of the major peaks of PHA and PWM responsive cells from regions of high to lower sedimentation velocity. Early after repopulation, PHA and PWM responsive cells were distributed over a wide range of overlapping sedimentation velocities.

In the immediate period (day 15) after repopulation, PHA responsiveness in the spleen was found exclusively in fast sedimenting cells. By day 20, the major peak of PHA responders was still at a region of high sedimentation velocity (>5mm/hr). On day 15 after repopulation, the sedimentation velocity of the major peak of PWM responsive cells in the different experimental groups varied: being 6 mm/hr in nTx->Tx mice, (Fig.21), 4.1 mm/hr in N->N mice (Fig.18), and a broad peak between 5 and 6 mm/hr in nTx->N

mice (Fig.19). By day 20, the major peak of PWM responsive cells in all groups had shifted to a region of sedimentation velocity less than 5mm/hr. After day 30, PHA and PWM responsive cells were found mainly in the region of medium sedimentation velocity (<5mm/hr), suggesting that maturation of mitogen responsive cells is accompanied by a decrease in cell size.

Contrary to the results obtained from unfractionated spleen cells, responsiveness to PHA was detectable in the fractionated spleens from all four experimental groups within 50 days of repopulation. This may be due to the enrichment of PHA responsive cells in certain fractions after cell separation.

Up to day 20 after repopulation, fast sedimenting PHA responsive cells were detectable in spleens of Tx recipients, although the overall activity was low (peak activity per spleen was 1.2×10^5 and 1.6×10^5 cpm/velocity increment for N->Tx and nTx->Tx animals respectively). Thereafter, no PHA responsiveness was detected.

4. Summary of Results

The results of this study show the following:

a) Humoral immune responsiveness to POL and SRC in irradiated, bone marrow repopulated mice recovered faster than responsiveness to PWM and PHA.

b) Thymectomy of bone marrow donors or recipients

impaired the recovery of the responsiveness to POL, a T-independent antigen.

c) Thymectomy of bone marrow recipients prevented the recovery of responsiveness to SRC and to PHA. The responsiveness to PWM was also only partially restored.

d) PHA responsiveness could be detected within 50 days of repopulation in fractionated but not in unfractionated spleen cells.

e) There was an overlap in the sedimentation velocity distribution profiles of PHA and PWM responsive cells in normal as well as restored animals.

f) During the course of development, the major peaks of mitogen responsive cells shifted from a region of high towards one of lower sedimentation velocity.

D. Discussion

In this study, humoral responsiveness to a T-dependent antigen, SRC, in irradiated, bone marrow grafted mice showed a slower rate of recovery than that against a T-independent antigen, POL. This is in agreement with the results presented in Chapter IV using mice reconstituted with fetal liver stem cells (Fig.3).

In N recipients, responsiveness to PHA, a T-cell mitogen, was restored later than that to PWM which can

stimulate both B- and T-cells. This may be due to the slower development of T-cells. If this is so, the responsiveness to PWM observed prior to the onset of PHA responsiveness might be due to PWM responsive B-cells. Alternatively, responsiveness to PWM or PHA may be functional characteristics of T-cells at different stages of maturation. Consistent with this is the finding of Byrd, van Boehmer and Rouse (1973) which shows that thymocytes of newborn mice can respond to PWM but not to PHA. The data presented here show that both PWM and PHA responsiveness developed later than SRC responsiveness in bone marrow recipients. Since responsiveness to SRC depends on the presence of helper T-cells (Miller & Mitchell, 1967; Miller & Osoba, 1967), the results suggest that the helper function of T-cells develops earlier than mitogen responsiveness.

These results are compatible with the possibility that there are 3 subpopulations of T-cells, each having PHA responsiveness, or PWM responsiveness, or helper function. A simpler alternative is that T-cells acquire different functional characteristics at various stages of maturation as has been suggested by Raff and Cantor (1971).

The role of the thymus in the development of responsiveness to antigens and mitogens was investigated. The recovery of anti-POL responsiveness was impaired when either bone marrow donors or recipients were thymectomized. This suggests that thymectomy may influence the development of B-cells since POL is a T-independent antigen (Diener,

O'Callaghan & Kraft, 1971). However, it is unclear how this effect may be brought about.

In accordance with studies by other workers, the data show that responsiveness to PHA and the production of PFC to SRC are entirely thymus dependent whereas responsiveness to PWM is partially thymus dependent. Thus irradiated, bone marrow repopulated Tx mice showed a complete loss of PHA responsiveness as well as of the ability to produce antibodies against SRC. Their PWM responsiveness, however, was only partially abrogated. In contrast to these results, a low degree of responsiveness to PHA was observed in certain fractions of spleens from Tx recipients during the first 20 days following bone marrow repopulation (Figs. 20 and 21). All of these PHA responsive cells were found in a region of high sedimentation velocity (>5mm/hr). These cells are perhaps PHA responsive T-cells present in the bone marrow inoculum as suggested by the work of Claman (1974) which showed that PHA responsive cells are present in the bone marrow of both nude and neonatally thymectomized mice although their number is lower than that found in heterozygotes (nude/+) or sham thymectomized littermate controls. He concluded that the maintenance of these bone marrow T-cells (as defined by their ability to respond to PHA) is thymus-dependent. Consistent with this view are the results of the present study which show a loss of PHA responsiveness in Tx recipients after day 20 of repopulation.

T- and B-cells are functionally heterogeneous populations (Roitt et al. 1969; Meuwissen, Stuttman & Good, 1969). Their subpopulations have been characterized on the basis of cell surface antigenic composition (Cerotinni & Brunner, 1967; Roitt et al. 1969; Raff and Owen, 1971a) or physical properties such as density (Shortman, 1968), size (Miller & Phillips, 1969) and physical adherence differences (Shortman et al. 1972). In this study, splenic lymphocytes at different stages of development were characterized on the basis of size differences and tested for responsiveness to PHA and PWM. The results suggest that the maturity of spleen cells is reflected by their size. Less mature cells are bigger and therefore have faster sedimentation velocities.

The data show that there is extensive overlapping in the sedimentation velocity distribution profiles of splenic PHA and PWM responsive cells. This could be interpreted as an indication that both PHA and PWM responsive cells have similar size distributions. Alternatively, the cells found in the overlapping region of sedimentation velocities may be double responders to both PHA and PWM. Shortman et al. (1973) have demonstrated a similar but smaller overlap in the density distribution profiles of splenic PHA and PWM responsive cells. They also found identical density distribution profiles in thymus cells responsive to PHA and PWM, suggesting that some cells can respond to both mitogens. It seems, therefore, that better separation of

splenic PHA and PWM responsive cells can be achieved by density differences.

Contrary to the results obtained from unfractionated spleen cells, PHA responsiveness was detectable in fractionated spleens within 50 days of repopulation. This may be due to the concentration of the PHA responsive cells in certain fractions as a result of cell separation. An alternative possibility is that there may be suppressor cells present in the lymphocyte population early in development, thereby inhibiting the response of potential PHA reactive cells. Presumably the suppressor cells and the reactive cells differ in size and would therefore be found in different fractions following cell separation. In the absence of the suppressor cells, the reactive cells were then able to respond. Later in development, there may be more potential reactive cells in proportion to suppressor cells, thus diluting out the inhibitory effect of the suppressor cells. Alternatively, suppressor cells may reside in sites other than the spleen. This hypothesis, though proposed to explain observations on the development of responsiveness to mitogens, may also be relevant in the development of responsiveness to antigens. Evidence has been presented that in adult animals suppressor cells may be responsible for the maintenance of some forms of tolerance (Gershon et al. 1972). A high frequency of suppressor cells in the young during the early stages of immune ontogenesis may account for the high susceptibility to tolerance

induction of the young including the establishment of self-tolerance.

CHAPTER VIII

CORRELATION OF RESULTS AND CONCLUSIONS

In this study, an experimental model has been used to investigate the ontogeny of the murine immune system based on the fact that single hemopoietic stem cells can reconstitute the cellular components of the immune system in lethally irradiated mice. During the development of this experimental model, evidence was obtained which indicates that stem cells from the yolk sac differ from those found in the fetal liver or adult bone marrow of mice. The primary difference between these stem cells is that yolk sac cells have a lower spleen colony forming potential and this limits their use for the production of single colonies. Since fetal liver stem cells have a high cloning capacity, they were used in the majority of the work. The developmental aspects of some functional parameters of murine immunocompetent cells were then followed in irradiated CBA mice. The functional parameters tested include: antigen recognition as measured by antigen binding to lymphocytes, humoral immune responsiveness, cell-mediated GvH reactivity and tolerance induction to transplantation antigens.

The antigens chosen to study the onset of antigen recognition and humoral responsiveness were POL, a T-independent antigen, and SRC, a T-dependent antigen. Thus, differences in the kinetics of B- and T-cell development could be observed. Immunologic recognition of POL appeared

after a period of 18 to 20 days of clonal expansion from a single stem cell. This figure is in keeping with an estimate made by Jerne (1970) of the time required for the generation of a complete antigen-recognition spectrum by somatic mutational events postulated to occur in a few germ-line structural genes coding for antibodies against histocompatibility antigens of the species. In contrast to the 18 to 20 days required to express the POL-binding potential, more than 30 days elapsed before RFC to SRC were evident. Differences in T-cell development relative to the ontogeny of B-cells may account for this discrepancy. Disparities in the sensitivity of the two assays used may be another explanation.

The ability to produce a humoral response to POL or to SRC developed at a slower rate than the appearance of ABC or RFC respectively. Two possibilities can be considered: (a) the small numbers of ABC or RFC are insufficient to generate a detectable immune response, or (b) there is a further maturation phase between the antigen binding capacity and the ability to synthesize antibodies. In the case of the anti-SRC response, there is also a likelihood that T-cells require a longer maturation period than B-cells.

To test the possibility that T-cell maturation is intrinsically slower than that of B-cells, T-cell function was measured in the form of GvH reactivity. There was a close correspondence in the rate of appearance of GvH and of anti-SRC activity. It was tentatively concluded that T-

cells were responsible for the delayed anti-SRC response relative to the anti-POL response although it is not certain if helper cells and killer cells mature at the same rate.

An important feature in immune ontogenesis is the generation of tolerance in the young. This has been studied using two models: (1) the heart allograft, and (2) the radiation chimera. The failure to develop tolerance to Balb/c heart grafts when transplanted into CBA recipients of cloned cells may have been due to the limited and localized source of antigens. In this case, lymphoid development of the repopulated animals may have taken place in the virtual absence of alloantigens. On the other hand, the internal environment of the chimeric CBF1 animal, being a rich and accessible source of Balb/c antigens, is most conducive to tolerance induction for the developing CBA cells. The generation of tolerance in the radiation chimera model was measured by the absence of anti-Balb/c activity in a GvH assay based on the ability of CBA immunocompetent cells to inhibit erythropoiesis of Balb/c marrow in irradiated Balb/c hosts.

It was further shown that the success of tolerance induction was improved when lymphoid cells were exposed to antigens earlier in their development. The data are compatible with the hypothesis that there is a tolerance susceptible period prior to the onset of immunocompetence during lymphoid maturation.

The development of responsiveness to mitogens was

slower than that to antigens in irradiated, bone marrow repopulated mice. This probably reflects differences in maturity between the cells responsive to mitogens and those responsive to antigens. In comparison to the results obtained from mice which were irradiated and repopulated with cloned cells, the recovery of humoral responses to both POL and SRC occurred later in bone marrow repopulated animals. This may be due to (a) different cell doses used; and/or (b) the presence of immunocompetent cells in the bone marrow (Lafleur, Miller & Phillips, 1972) but not in spleen colonies (Nekori & Feldman, 1965). These cells are found to be incapable of giving rise to new immunocompetent cells, thereby reducing the total number of immunocompetent cell precursors in the original bone marrow inoculum.

The role of the thymus during the development of mitogen and antigen responsiveness was investigated. The results confirm the thymus dependence of the anti-SRC response and the responsiveness to PHA and PWM. It was also found that neonatal thymectomy of bone marrow donors impaired the subsequent expression of B-cell function as judged by the incomplete restoration of both the anti-POL and anti-SRC responses.

Within the first 50 days of bone marrow repopulation, responsiveness to PHA was detectable in certain fractions of spleen cells after separation. This observation is compatible with the notion that suppressor cells are present in the spleen during the early period of ontogeny, and their

presence may account for the high susceptibility to tolerance induction of the young.

Cells responding to PHA and PWM were shown to change their physical characteristics during the course of lymphoid development. Early in development, these cells had a high sedimentation velocity (>5mm/hr), and as maturation proceeded there was a concomitant shift towards a region of lower sedimentation velocity. Thus, mitogen responsive cells became smaller as they matured. Since considerable overlap occurred in the sedimentation velocity distribution profiles of PHA and PWM responsive cells, size differences alone are probably insufficient to physically characterize mitogen responsive cells. Furthermore, the fact that both PHA and PWM can stimulate T-cells could account for some of the observed overlap in sedimentation velocity distribution profiles.

Immune ontogenesis in CBA mice may be regarded as consisting of a sequence of events. Potentially immunocompetent cells are first found in the yolk sac and the first step in development is marked by the appearance of pluripotent stem cells which reside in both fetal as well as adult hemopoietic tissues. These multipotential stem cells can form spleen colonies in irradiated recipients and they exhibit similar characteristics regardless of their origin. The next step in development involves proliferation and differentiation of these cells to produce a population of lymphocytes heterogeneous in physical, antigenic and

functional characteristics. Lymphocyte differentiation can occur along two different pathways. The development of immunological functions in a population of lymphocytes begins with antigen recognition. This is followed by humoral responsiveness and cell-mediated immune reactivity. Since immune responses involve cell interactions, this order of appearance of immunological functions reflects the sequence of maturation of the limiting cell type in an immune response. The results of this study are in agreement with the contention that B-cells develop at a faster pace than T-cells.

An important aspect in the development of immunocompetent cells is the acquisition of specific unresponsiveness to self antigens. This process seems likely to occur during the interval between the development of antigen recognition and the ability to respond to specific antigenic stimulation. Perhaps responsiveness in the young is at first inhibited by suppressor cells, thus resulting in a high susceptibility to tolerance induction and the establishment of self-tolerance.

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(2) Ontogeny of the immune system: generation of clonal diversity. Canad. Fed. Biol. Sci. (Abstract) 1972.

(3) Recognition of a self antigen: antigen binding lymphocytes in normal man and guinea pig to human encephalitogenic protein. J. Immunol. 110: 1383, 1973.

(4) Ontogeny of the murine immune system: development of antigen recognition and immune responsiveness. Europ. J. Immunol. 3: 224, 1973.

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